Differential Roles for the Retinoblastoma Protein in Cycling and Quiescent Neural Populations

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ABSTRACT

While the genetics of retinoblastoma and the implications of the retinoblastoma susceptibility gene, *RB1*, are well described, there is still scarce evidence to suggest why *RB1* acts in such a cell-type specific manner. Using the *murine* cortex as a model, we examined the effects of *RB1* deletion of cycling neural progenitors and post-mitotic neurons, in order to ascertain cell-type specific functions in the central nervous system. Using the previously identified cell-cycle independent role for Rb in tangential migration, we validated Rb/E2f regulation of neogenin and implicated it in this process. In quiescent cortical neurons, we identified a pivotal role for Rb in neuronal survival. Unlike in cycling progenitors, in post-mitotic neurons Rb specifically represses the expression of cell-cycle associated genes in an E2f-dependent manner. Finally, in cortical neurons in the absence of Rb, we observe an activation of chromatin at E2f associated promoters. To determine the role of direct interaction between Rb and chromatin modifying enzymes, we utilized an acute LXCXE-binding deficient mutant paradigm. We report that the LXCXE binding motif is dispensable in establishment and maintenance of cortical neuron quiescence and survival. The activation state of E2f-responsive promoters appears to be dependent on E2f-activity and not simply Rb-mediated repression. Taken as a whole, this thesis serves to support the hypothesis that Rb plays a diverse role in different cell-types by regulation of unique gene targets and regulatory mechanisms. Characterizing specific cancer-initiating populations and understanding the specific function of Rb will help in the treatment of many cancers resulting from *RB1* mutation or mutation within the Rb/E2f pathway.

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LIST OF ABBREVIATIONS

14-3-3σ	14th fraction of bovine brain homogenate, found on positions 3.3 of electrophoresis
3D	three dimensional
5'	fifth carbon in sugar-ring of nucleic acid, region upstream of gene
AC-3	active caspase 3
Acetyl-H3	acetylated histone 3
AD	Alzheimer's disease
ANOVA	analysis of variance
Apaf1	apoptotic protease activating factor 1
APC/C	anaphase-promoting complex/cyclosome
ApoE	Apolipoprotein E
ATG	start of protein translation
ATM	Ataxia telangiectasia mutated
ATP	adenosine triphosphate
AurA	aurora kinase A
BH-3	Bcl-2 homology domain
Brca1	breast cancer 1
BrdU	bromodeoxyuridine
Brg1	brahma related gene 1
BSA	bovine serum albumin
°C	celsius
C57Bl/6	C57 black 6
Cap-D3	chromosome associated protein D3
Ccna2	encoding cyclin A2
CD1®	Institute for Cancer Research outbred mice
Cdc2	cell division cycle homolog 2
Cdc25a	cell division cycle homolog 25 a
Cdk	cyclin dependant kinase
CDKI	cyclin dependant kinase inhibitor
ChIP	chromatin immunoprecipitation
Chip	microarray chip
Chk	checkpoint kinase
Chx10	*C. elegans* ceh-10 homeodomain containing homolog
CIHR	Canadian Institutes of Health Research
Cip/Kip	Cdk interacting protein/ kinase inhibitory protein
CNS	central nervous system
Cond	conditional
Cre	cyclization recombination enzyme
CtBP	carboxy terminal binding protein

C-terminal	carboxy
CycA2	cyclin A2
D. melanogaster	*Drosophila melanogaster*
Da	Dalton
DAVID	Database for analyzation, visualization and integrated discovery
DCC	deleted in colorectal cancer
DIV	days in vitro
DKO	double knock out
Dlx	distaless related homologue
DNA	deoxyribonucleic acid
DOC	deoxycholate
DP	dimerization partner transcription factor
DREAM	*Drosophila* RBF, E2F and Myb-interacting protein complex
DTT	1,4-dithiothreitol
E	embryonic day
E1A	adenovirus early region 1A
E2F	E2 promoter binding factor
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol tetra-acetic acid,
EMMA	European Mouse Mutant Archive
EMSA	electrophoretic mobility shift assay
ERT2	estrogen receptor T2
FANC	Fanconi anemia
FDR	false discovery rate
FGF	fibroblast growth factor
Fig	figure
flox	flanked loxP sites
Foxg1	forkhead box G1
FVB/N	Friend virus B type susceptibility/ NIH mouse
G_0	gap 0 (quiescence)
G_1	gap 1 (interphase)
G_2	gap 2 (interphase)
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GO	gene ontology
GTP	guanosine tri-phosphate
h	hour
γH2Ax	serine 139 phosphorylated histone variant H2Ax
H3K9me3	trimethylated histone 3 on lysine 9
H4K20	histone 4 lysine 20
H3K4me3	trimethylated histone 3 lysine 3

HBSS	Hank's balanced salt solution
HDAC	histine deacetylase
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hes	hairy and enhancer of split homolog
Hh	hedgehog
HP1	heterochromatin protein 1
HPV	human papilloma virus
HSFO	Heart & Stroke Foundation of Ontario
Id2	inhibitor of differentiation 2
Ink4	inhibitors of cdk4
IP	immunoprecipitation
IgG	immunoglobulin G
ISH	in situ hybridization
IZ	intermediate zone
k	kilo
L	litre
ΔL	LXCXE-binding disruption mutant
LGE	lateral ganglionic eminence
loxP	locus of X-over P1
Lhx6	LIM homeobox gene 6
LXCXE	lysine- any amino acid- cysteine- any amino acid- glutamic acid
M	molar
m	micro
M phase	mitosis
m_	milli
_m	metre
µ	micro
MAP	microtubule associated protein
MB	marked box domain
Mb	mega base
MEA	modular enrichment analysis
MEF	mouse embryonic fibroblast
MGE	medial ganglionic eminence
mGlu4	metabotropic glutamate receptor 4
min	minute
MLL	myeloid/lymphoid or mixed lineage leukemia
MOI	multiplicity of infection
Mre11	meiotic recombination 11 homolog 1
mRNA	messenger RNA
mut	mutant

myb	V-myb myeloblastosis viral oncogene homolog (avian)-like 2
myc	myelocytomatosis viral oncogene
MyoD	myogenic determination factor
MZ	marginal zone
N	haploid chromosome number
n	nano
n=	number
Nbs1	nibrin
NeuN	neuronal nuclei
Nf1	neurofibromin 1 or Neurofibromatosis type I
NGF	nerve growth factor
Nkx2.1	Nk2 homeobox 1
NMDA	*N*-Methyl-D-aspartic acid
NP-40	nonidet P-40
N-terminal	amino
Noxa	noxious stresses
OB	olfactory bulb
OGS	Ontario Graduate Scholarship
OGSST	Ontario Graduate Scholarship in Science and Technology
Orc	origin recognition complex
P	probability value
P19	pluripotent embryonal carcinoma cell line
Pax6	paired box gene 6
PBS	phosphate buffered saline
PC12	pheochromocytoma cell line
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC1α	peroxisome proliferator-activated receptor- coactivator (PGC)-1
pH	potential of hydrogen
PH3	phosphohistone 3
PNET	primitive neurectodermal tumour
PNS	peripheral nervous system
PP1c	protein phosphatase 1c
PPARγ	peroxisome proliferator-activated receptor
PSD95	post-synaptic density 95kda
PTEN	phosphatase and tensin homolog
Puma	p53-upregulated modulator of apoptosis
Prox1	prospero homeobox protein 1
Prx1	periaxin 1
Ptch	patched

qRT-PCR	quantitative real-time polymerase chain reaction
Ras	rat sarcoma
Rad50	recombination repair protein 50
RB1	retinoblastoma gene
Rb	retinoblatoma protein
Rbbp2	Retinoblastoma binding protein 2
RGM	repulsive guidance molecule
RMA	robust Multi-Array average
RNA	ribonucleic acid
Robo	roundabout protein
RT	room temperature
Runx2	runt-related transcription factor 2
S phase	DNA synthesis
SAM	signifiance analysis of microarray
Saos-2	sarcoma osteogenic
SD	standard deviation
SEM	standard error of the mean
Sema 3	class 3 semphorin
SGZ	subgranular zone
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIVA	SIVA apoptosis inducing factor 1
Skp2	S-phase kinase-associated protein 2, the F-box protein of the SCF complex
Smo	smoothened
Suv39h1	suppressor of variegation 3-9 homolog 1 (Drosophila)
SV40	Simian Virus 40
SVZ	subventricular zone
SWI/SNF	switching/sucrose non-fermenting
TAD	transactivation domain
TAM	tamoxifen
Tris	tris(hydroxymethyl)aminomethane
Twist	twist gene homolog 1 (Drosophila)
VZ	ventricular zone
Wnt	wingless, integration 1

CHAPTER 1- INTRODUCTION

1.0.1 Neurogenesis

The mammalian nervous system is arguably the most complex tissue system, composed of a broad array of cell classes which can be further subdivided into an almost limitless set of sub-types (reviewed in Gotz and Huttner, 2005; Nguyen et al., 2006b). The process of neurogenesis, whereby an immature neural precursor cell (stem and/or progenitor) differentiates into a mature neural cell (Figure 1.1), is tightly regulated by a number of autonomous and non-autonomous factors (Gotz and Huttner, 2005; Nguyen et al., 2006b). These factors give a cell not only the capacity to differentiate, but to regulate processes such as long-distance migration, survival and establishment of projections (Gotz and Huttner, 2005; Nguyen et al., 2006b).

The developing mammalian cortex is composed of two primary neurogenic niches (Figure 1.1) (Gotz and Huttner, 2005; Nguyen et al., 2006b). The ventral niche, referred to as the ganglionic eminence, predominantly gives rise to inhibitory interneurons (Parnavelas et al., 1991) during early to mid-neurogenesis (Olsson et al., 1998), and switches to glial cell output at the later stages of embryonic neurogenesis (Kessaris et al., 2006; Menn et al., 2006). Interneurons born in the ganglionic eminence initiate a differentiation program controlled by combinatorial expression of homeodomain-containing transcription factors (*Nkx2.1*, *Lhx6*, *Dlx1/2*) (reviewed in Wonders and Anderson, 2006), and migrate tangentially to the dorsal cortex (reviewed in Kriegstein and Noctor, 2004). Neurons born in the dorsal niche consist of excitatory projection neurons (Parnavelas et al., 1991). In the dorsal niche, cells born along the ventricle migrate radially and populate the cortex in an inside-out manner, where the deeper layers are formed prior to the superficial layers (Berry and Rogers, 1965; Rakic, 1974). A unifying theme in the differentiation and integration of

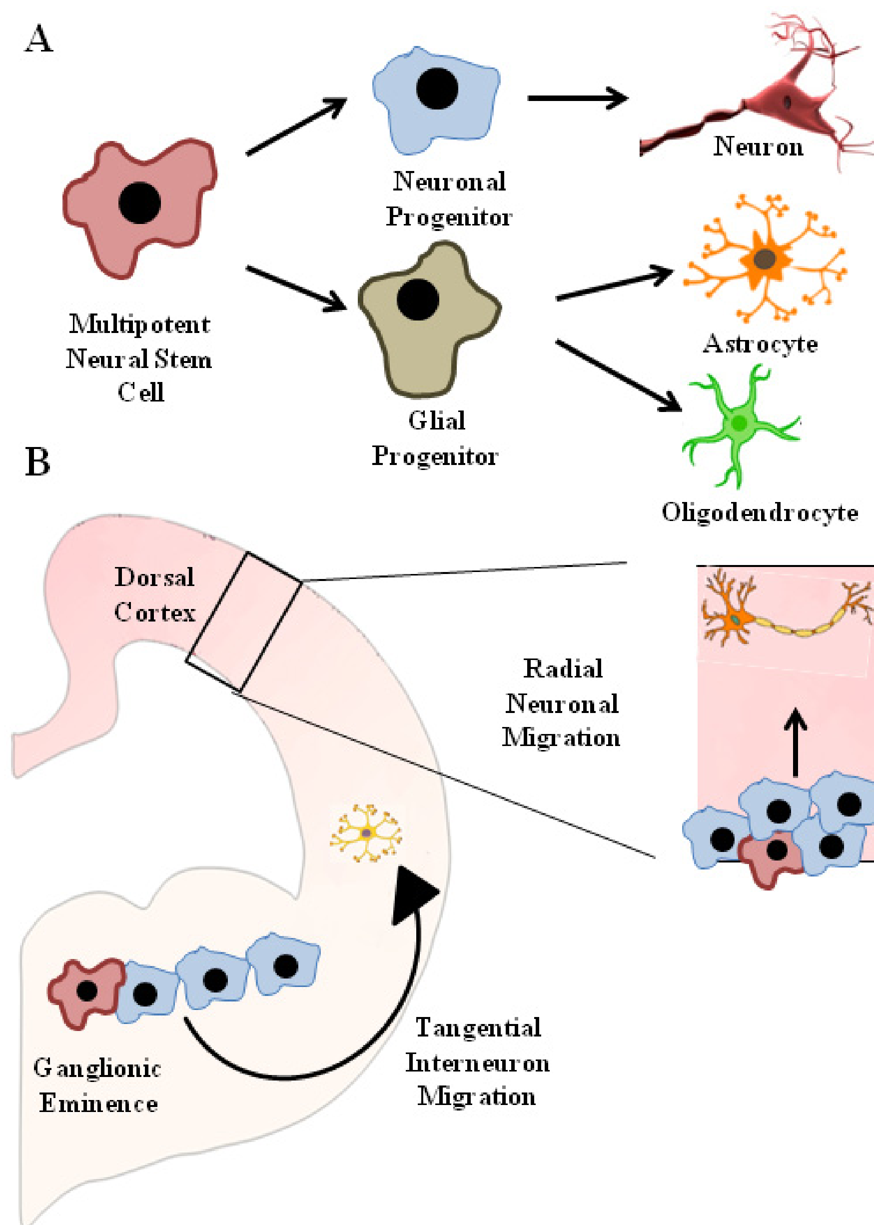

Figure 1.1 Mammalian cortical neurogenesis. A) Multipotent neural stem cells are able to give rise to committed neuronal or glial progenitors. These progenitors are able to differentiate into neurons or astrocytes/oligodendrocytes, respectively. B) Schematic of neuronal migration in the developing forebrain. Ventrally derived progenitors from the ganglionic eminence migrate tangentially and differentiate into interneurons. Dorsally-derived progenitors migrate radially and give rise to projection neurons

Figure adapted from (Yokota et al., 2007)

precursors from each cortical niche is the interplay between autonomous and non-autonomous factors to govern proper output (reviewed in Hebert, 2005). The integration of all these pathways in a spatial and temporal fashion is integral for proper nervous system development (Hebert, 2005). Perturbations in any of these pathways, however subtle, can result in developmental disorders ranging from epilepsy to lissencephaly (reviewed in Dixon-Salazar and Gleeson, 2010; Fung et al., 2012). Dissecting the molecular events that underlie normal neural developmental homeostasis is pivotal in our understanding of how these events are altered in various disease states.

The implementation of large scale genomic and proteomic techniques have allowed us to gain a significant amount of insight into the development and maintenance of the nervous system (Hu-Lince et al., 2005). An emerging paradigm in nervous system homeostasis is the multi-faceted role for the cell cycle machinery (reviewed in Hebert, 2005). Initially thought to only regulate the events of cell division, it is now being appreciated that these factors play complex and diverse roles throughout many stages of nervous system development (reviewed in Herrup and Yang, 2007). **The central theme of this thesis is the diverse role that the cell cycle regulatory protein, Rb, plays in different neural populations. To better understand these roles, the function of Rb was examined in proliferating neural precursor cells and quiescent post-mitotic cortical neurons. The goal of this introductory chapter is to emphasize the tissue and cell-type specific roles for cell cycle regulatory factors. Furthermore, I will highlight established physiological and molecular events controlled by Rb and the questions which remain to be answered with regard to its role in the nervous system.**

1.0.2 Cell Cycle Control – An Overview

In order to gain an understanding of the diverse role that cell cycle regulators play in the development and maintenance of the brain, it is essential to appreciate their primary role: regulation of the cell cycle. The original dogma, proposed in 1858 by Rudolf Virchow, that new cells must arise from established cells laid the philosophical groundwork for discovery of the cell cycle. The cell cycle is the step-wise reproduction of cellular constituents and division suggested by Virchow. Initial studies in unicellular budding and fission yeast identified the basic cell cycle machinery (Cyclins and Cyclin-dependent kinases(CDKs) though the complexity of cell division is obviously much greater in multi-cellular eukaryotes (Figure 1.2) (Lee and Nurse, 1987), and reviewed in McClellan and Slack, 2006). The following will provide an overview of the basic mammalian cell cycle machinery and its role in nervous system function. Emphasis will be placed upon classical cell cycle regulators that play a direct role in influencing cell cycle progression.

1.1 Regulation of Interphase of the Cell Cycle

1.1.1 G_1 and S Phases

The initial phase of the cell cycle, G_1 or the gap phase 1, serves as a resting period between multiple rounds of cell division (reviewed in Lange and Calegari, 2010; Salomoni and Calegari, 2010). Once a cell decides to progress from G_1 to the S phase of the cell cycle, the G_1/S restriction point must be satisfied (Figure 1.2). Restriction points exist in the cell cycle to ensure proper genomic fidelity during cell division, with improper cell division triggering programmed cell death or even pathological events such as cancer (reviewed in Sperka et al., 2012). The G_1/S transition is controlled by the Rb/E2f pathway which will be

discussed at length later in this introduction and therefore will briefly be introduced here (reviewed in Burkhart and Sage, 2008; Chen et al., 2009). The length and timing of G_1 is dependent on many extracellular and environmental cues (reviewed in Blagosklonny, 2007). A cell in non-favourable mitotic conditions will increase G_1 length or even permanently arrest (G_0) until a time that division is appropriate (Blagosklonny, 2007).

The activation state of G_1/S restriction point is dependent on the interplay between activators (CDKs) and inhibitors (cycle dependent kinase inhibitors (CDKi) (reviewed in Malumbres and Barbacid, 2009). Induction of proliferation by mitogenic stimuli, such as growth factors, results in the formation of a CDK-Cyclin complex, enabling its kinase activity (Booher and Beach, 1987; Draetta et al., 1989; Koff et al., 1992; Xiong et al., 1992). Conversely, non-mitogenic conditions up-regulate CDKi's, which are able to arrest cell cycle progression by antagonizing CDK/Cyclin activity (reviewed in Besson et al., 2008). CDKi's include factors from the Ink4a/Arf and Cip/Kip families (Besson et al., 2008). Active CDK6/Cyclin D and CDK4/Cyclin D phosphorylate Rb, relieving its inhibition of E2f, during G_1 (Ewen et al., 1993; Kato et al., 1993; Serrano et al., 1993). E2f's are a class of transcription factors involved primarily in the regulation of core cell cycle genes, most importantly those involved in DNA synthesis and progression to S-phase of the cell cycle (Johnson et al., 1993). An important E2f target, Cyclin E, is up-regulated allowing sufficient CDK2/Cyclin E for further phosphorylation of Rb, maximal cellular E2f activity and progression to S-phase (Botz et al., 1996; Koff et al., 1991; Koff et al., 1992; Obeyesekere et al., 1995). The cell now possesses all the core machinery to begin S-phase and DNA synthesis.

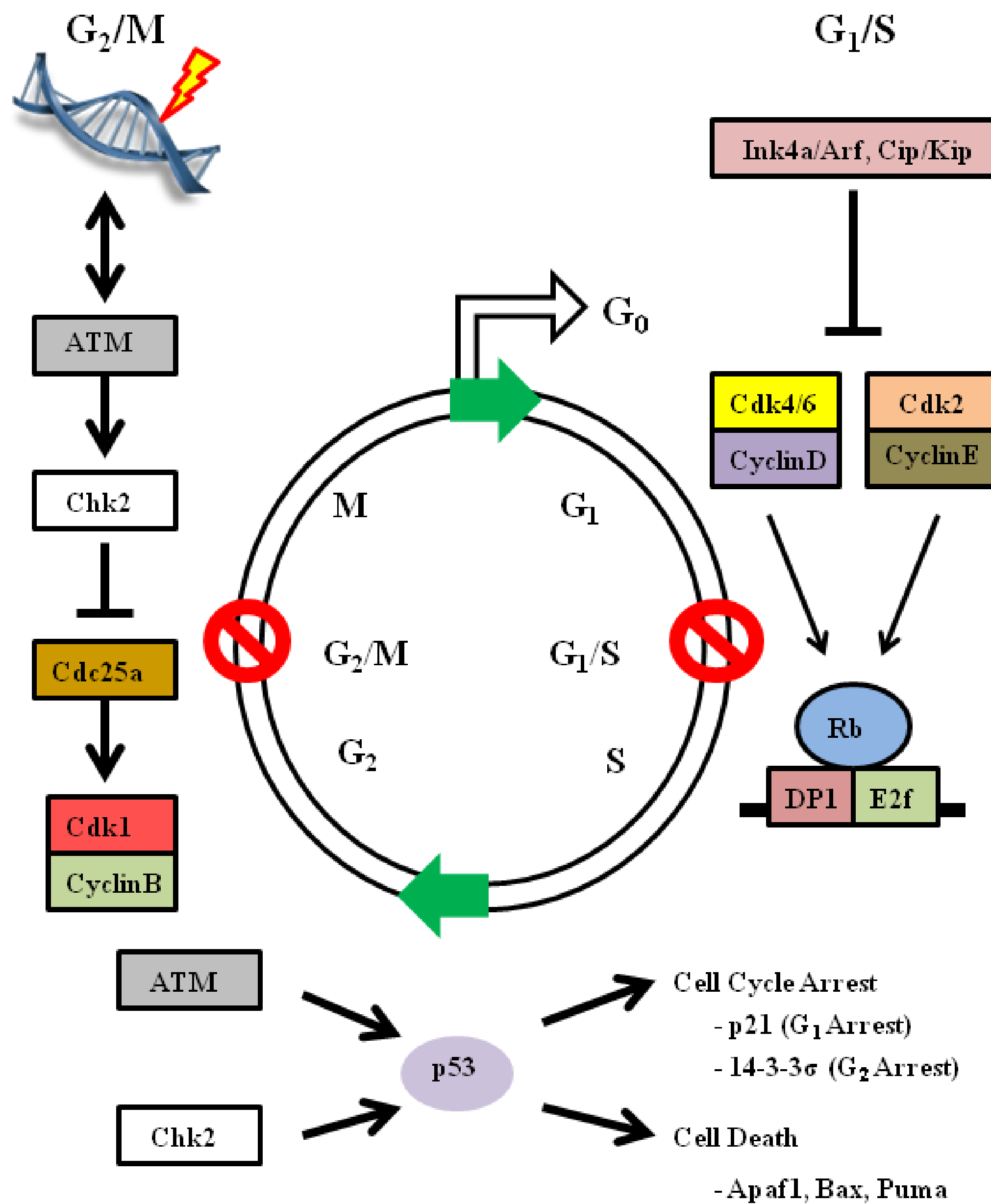

Figure 1.2 Mammalian G1/S and G2/M cell cycle checkpoints. CDK/Cyclin activity drives the progression from G1 to S-phase by phosphorylation of Rb. Rb phosphorylation de-represses the E2f transcription factor, resulting in upregulation of S-phase associated genes. In the absence of DNA damage, Cdc25a de-phosphorylates and actives CDK1/CyclinB allowing progression to M-phase. In the presence of DNA damage, sequential activation of ATM and Chk2 triggers G2 arrest by inhibiting Cdc25a. DNA damage and G2 arrest activates p53 which can trigger further cell cycle suppression by up-regulation of p21 and 14-3-3σ, or cell death by transcriptional up-regulation of apoptotic factors.

S-phase of the cell cycle is driven by CDK2/Cyclin E and CDK2/Cyclin A complexes (reviewed in Symeonidou et al., 2012). The most imperative factor in proper S-phase progression is the faithful re-production of the genome, which is ensured by several mechanisms. During S-phase, DNA polymerase is recruited to pre-replication complexes (origin replication complexes) formed in G_1 by the interplay of many factors including CDK2/CyclinE and CDK2/CyclinA (Symeonidou et al., 2012). The recruitment of DNA polymerase results in the replication of a single copy of the genome. Once a single replication site has undergone duplication, further DNA re-replication is prevented by the recruitment and phosphorylation of replication complexes by S-phase CDK/Cyclin's (Symeonidou et al., 2012). To ensure proper quality control, damage and repair pathways act at an intra-S-phase checkpoint to ensure proper genome duplication prior to progression to G_2 (reviewed in Bartek and Lukas, 2001; Pichierri and Rosselli, 2004). The upstream mechanisms of intra-S-phase arrest parallel those of G_2/M arrest; however, ultimately result in the inhibition of DNA synthesis (Bartek and Lukas, 2001).

1.2.2 G_2 and G_2/M

The second gap phase of the cell cycle, G_2, exists to ensure that the genome duplication that occurred during S-phase proceeded without error. The G_2/M checkpoint is vital in ensuring genome quality, as this is the final point to verify integrity prior to division and prevent mitosis in the presence of DNA damage (Figure 1.2). In the absence of DNA damage, CDK1/Cyclin B complexes act on a number of cellular substrates to initiate progression to M-phase (mitosis) (reviewed in Lindqvist et al., 2009). In the presence of DNA damage, a number of mechanisms are in place to prevent the activity of CDK1/Cyclin B and other components required for M-phase entry (Lindqvist et al., 2009).

There are many forms of DNA damage and thus many mechanisms of damage recognition and repair (reviewed in Sperka et al., 2012). Double strand breaks will be used as an example of damage, as it is most relevant to this thesis. In the context of double strand DNA breaks, the site of DNA lesion is recognized by a trimeric protein complex containing Mre11, Rad50 and Nbs1 (Carney et al., 1998). This complex recruits the serine/threonine protein kinase, ATM (Lee and Paull, 2004), which is activated through a series of autophosphorylation and adaptor protein binding events (reviewed in Derheimer and Kastan, 2010). ATM phosphorylates the key effector, Chk2, which subsequently phosphorylates and de-activates the phosphatase Cdc25a (Derheimer and Kastan, 2010). Cdc25a activates the CDK1-Cyclin B complex by removing inhibitory phosphate groups; however, this does not occur upon ATM-Chk2 activation (Derheimer and Kastan, 2010). ATM and Chk2 can induce upstream cell cycle arrest by stabilizing of p53 and induction of the G_1/S CDKi p21 (Derheimer and Kastan, 2010; Jack et al., 2002; Takai et al., 2002). A similar cascade can be initiated and propagated by the ATM-Chk2 related ATR-Chk1 pathway (reviewed in Smith et al., 2010). Sustained or irreparable DNA damage results in a prolonged response causing programmed cell death via transcriptional up-regulation of the apoptotic machinery (Apaf1, Puma, Noxa) by p53 (reviewed in Bieging and Attardi, 2012). Apoptosis represents the extreme lengths a cell will take in order to prevent genomic damage. Mutations in G_2/M and DNA damage sensing pathways are common in many familial and sporadic forms of cancer (*BRCA1*, *ATM*, *FANC*) (reviewed in Lobrich and Jeggo, 2007). The redundancy in checkpoint and cell-cycle inhibitory pathways signifies the paramount importance of maintaining genomic integrity prior to M-phase entry.

Once the genome quality has been verified at the G_2/M checkpoint, the cell commits to undergoing M-phase of the cell cycle. Much like interphase (G1-S-G2), M-phase consists of a series of regulated step-wise events in order to increase proper mitotic fidelity and chromosomal segregation (reviewed in Vleugel et al., 2012). Proper chromosomal segregation ensures genetic homeostasis in cellular progeny. Defects in chromosomal segregation result in localized DNA damage or in more extreme cases aneuploidy, where a daughter cell inherits a chromosomal number greater or less than 2N (reviewed in Schvartzman et al., 2010). Aneuploidy is considered to be a precursor to many malignancies and is also involved in trisomy disorders (Schvartzman et al., 2010). Due to the limited space available, reviews of M-phase regulation can be referenced here (Kimble, 2011; Malumbres and Barbacid, 2009; Vleugel et al., 2012), as further discussion is beyond the scope of this thesis.

1.2.3 G_0

A final, often overlooked, phase of the cell cycle is G_0. G_0 represents a period of sustained cell-cycle repression. A normal physiological G_0 state, quiescence, is generally associated with a terminal post-mitotic differentiated state in cells such as neurons. In certain cell-types, such as hepatocytes, exit from G_0, re-entry into the cell cycle and proliferation is observed (Klochendler et al., 2012; Shea, 1964). G_1 and G_0 both represent periods of sustained CDK inactivity; however, in a G_0 state, cells will generally become refractory to mitogenic stimuli, such as growth factors, that previously elicited a proliferative response in their G_1 state (Konigsberg et al., 1960).

There is evidence that most developmental G_0 induction is reversible (Blais et al., 2007; Campa et al., 2008), even in post-mitotic neurons (Kruman et al., 2004); however, stress-induced G_0 entry, referred to as senescence, is seen as a permanent removal from the cell cycle (reviewed in Acosta and Gil, 2012). Senescence has been observed in a number of stress-induced scenarios (Acosta and Gil, 2012). The induction of senescence in aging stem and progenitor populations has largely been attributed to the stress-associated with telomere shortening and subsequent DNA damage (reviewed in Sperka et al., 2012). In order to permanently arrest proliferation to prevent damage to progeny, G_0 is induced by a coordinated Rb/p53 mediated response (Hiyama et al., 1995; Shay et al., 1991). Many of the steady-state cell cycle checkpoints are coordinately activated to induce senescence in numerous models (Sperka et al., 2012). Furthermore, unlike normal quiescence-based G_0, senescence results in a hallmark deposition of the repressive histone mark H3K9me3, highlighting its fundamental differences and permanence when compared with quiescence (Lezhava, 1984; Prashad and Cutler, 1976). The contrast between developmental and stress-induced G_0 highlights the complexity of this seemingly simplistic state of cell-cycle withdrawal.

1.3 Neural Precursor Cell Cycle Regulation and Dynamics

The cell cycle, though following a similar progression of cyclical CDK-Cyclin activation/deactivation, differs from tissue to tissue (reviewed in Gopinathan et al., 2011). These differences are underscored by the compensatory capacity observed in Cyclins and CDK's (Gopinathan et al., 2011). Many of these factors have been shown to moonlight in other capacities that deviate from their classical roles in cell division (reviewed in Frank and Tsai, 2009; McClellan and Slack, 2006).

The most paradoxical studies examining CDK and Cyclin function highlight their dispensability under most contexts. Germline knockout mice for CDK1 exhibit early embryonic lethality (Santamaria et al., 2007); however, other interphase CDK's do not share this same importance. The genetic loss of both CDK4/6 in a *murine* model, though resulting in embryonic lethality, displayed normal organogenesis and proliferation (Malumbres et al., 2004). Animals lacking CDK2, which was hypothesized to be essential in the G_1/S transition, develop normally and display no changes in viability (Berthet et al., 2003). Further studies revealed that these animals were sterile, owing to a specific defect in meiotic division (Berthet et al., 2003). In depth examination of proliferation of adult subgranular zone neural precursor cells null for CDK2 showed a dispensable role for the protein in normal and pathological conditions (Vandenbosch et al., 2007). In contrast, loss of CDK2 in adult subventricular zone neural precursors disrupted proliferation, highlighting the cell-type specific role, in adult neurogenic niches, for CDKs (Jablonska et al., 2007). Perhaps owing to their similar developmental origin as adult SVZ precursors, progenitors from GE-derived precursors expressing a dominant negative CDK2 induced growth arrest *in vitro* (Ferguson et al., 2000). The differential roles for CDK2 in two similar niches within the brain reinforce the specificity of cell-cycle regulatory factors within tissue and progenitor populations. Due to their limited redundancy, CDK's exhibit less functional redundancy than their Cyclin binding partners.

Cyclins display a more complex pattern of expression than their cognate CDK binding partner. Not only do multiple Cyclins exist within the same family, alternative CDK binding partners can compensate for lost expression. This is most elegantly highlighted in the study by Geng Y et al., in which they demonstrate knocking human Cyclin E into the

mouse Cyclin D1 locus is sufficient to recapitulate expression and function of the protein (Geng et al., 1999). Due to this redundancy, many animals do not display significant defects upon removal of individual Cyclins although; in-depth tissue specific analysis has revealed unique roles for Cyclins in cell cycle regulation.

The function of many Cyclins may be redundant; however, many interesting non-over-lapping roles have been shown for specific Cyclins in the nervous system and non-CNS tissues. Though their expression has been implicated in upstream processes that drive neural proliferation such as the Hh and Wnt pathways (reviewed in Fuccillo et al., 2006; Niehrs and Acebron, 2012), no studies have specifically examined the role of E, A or B type Cyclins in neural precursor cells. Examination of conditional mutants for both Cyclins A1 and A2 showed they were not essential in proliferation of fibroblasts due to compensatory Cyclin E expression (Kalaszczynska et al., 2009). In the absence of both A and E type Cyclins, fibroblasts displayed a complete growth arrest (Kalaszczynska et al., 2009). This was not the case in embryonic stem cells and haematopeiotic cells, where ablation of Cyclin A expression impeded cell cycle progression (Kalaszczynska et al., 2009). This paralleled studies examining complete loss of Cyclin D (D1-D3), in which fibroblasts proliferated to near wild-type levels in their absence yet, a similar dependence was observed in the hematopoietic lineage (Kozar et al., 2004). Specific analysis of the adult neurogenic niches, revealed a specific requirement of Cyclin D2 (Kowalczyk et al., 2004). Animals deficient in D2 displayed reduced proliferation, whereas D1 knockouts had no significant defects (Kowalczyk et al., 2004). Furthermore, perturbations in G_1 Cyclin expression have been shown to modulate neurogenic output during development due to altering the length of S-phase (Pilaz et al., 2009). Decreasing the length of S-phase was shown to lead to an increase

in neuronal production (Pilaz et al., 2009). These studies highlight the complexity and over-lapping roles exhibited by Cyclins during cell division in distinct tissue types. In addition to their function in cell division, unique roles for Cyclins have been described outside of cell cycle regulation.

Dissociating the cell cycle and non-cell cycle functions of core cell cycle regulatory proteins is often difficult due to the proliferation phenotypes associated with their removal. Several lines of evidence have shown unique non-cell cycle roles for these factors in the nervous system. In differentiating motor-neurons, Cyclin D1 expression is extended beyond that of Cyclin D2 (Lukaszewicz and Anderson, 2011). Analysis of the role of Cyclin D1 in these cells revealed a specific non-cell cycle role in the regulation of differentiation through an epistatic interaction with Hes6, a proneurogenic transcription factor (Lukaszewicz and Anderson, 2011). Cyclin D2 localization was shown to dictate fate-choice in neural progenitors by governing asymmetric division (Tsunekawa et al., 2012). In a similar fashion in *D. melanogaster*, Cyclin E was shown to regulate fate-decisions in the developing nervous system by regulating the switch from symmetric to asymmetric cellular division. This was shown to occur independently of cell cycle activity and dependent on regulation of the Prospero, *Prox1* in mammals (Berger et al., 2010). These studies support recent results that perturbations in G_1 length are able to affect fate-decisions in the nervous system. Decreasing G_1 length by over-expression of CDK4/Cyclin D1 was shown to expand the pool of adult neural precursor cells, suggesting a similar importance to that shown in embryonic precursors (Artegiani et al., 2011; Lange et al., 2009). Moreover, the absence of CDK4/Cyclin D1 induced a prolonged G_1 period in SGZ neural precursors resulting in a decrease precursor pool (Lange et al., 2009). These studies suggest that the neurogenic

effect of manipulation of the cell cycle machinery is dependent on cell-cycle function, as opposed to the aforementioned works. Dissociating cell cycle and non-cell cycle properties is difficult in proliferating populations, which contributes to the controversial role for many of these factors. Delineating specific non-cell cycle pathways becomes more apparent when cells become post-mitotic.

1.4 Cell Cycle Regulators in Post-Mitotic Neurons

Perhaps the most unexpected result with regard to the function of the cell cycle machinery came with the observation that many of these factors are expressed in cell types that no longer participate in the cell cycle (reviewed in Frank and Tsai, 2009; McClellan and Slack, 2006). From these observations, a better understanding of the complexity of many of these factors has been realized, in both neuronal and non-neuronal cell types, impacting the way we view cell-cycle dysfunction in many pathological states (reviewed in Chen et al., 2009; Herrup and Yang, 2007; Rashidian et al., 2007).

A wide array of cell cycle regulators from G_1-M-phases of the cell cycle have been implicated in non-cell cycle functions in post-mitotic cell types. An initial observation in various post-mitotic cell types, including cardiomycocytes and neurons, noted that various origin recognition complex proteins (Orc) were expressed and immunoprecipitated with non-Orc factors (Thome et al., 2000). The Orc complex is primarily involved in the formation of the replication origins required for DNA synthesis (reviewed in Symeonidou et al., 2012). Further analysis revealed a specific localization of Orc subunits to dendrites in several neuronal subtypes (Huang et al., 2005). siRNA mediated knockdown of Orc subunits led to significant impairment in dendritic growth and branching while expression of the Orc

ATPase subunit increased arborisation (Huang et al., 2005). Subsequent studies found that in cultured cerebellar granule neurons, Orc3 expression correlated with the mature neuronal markers MAP-2, PSD-95 and PHCCC (Cappuccio et al., 2010). This effect was shown to be due to the modulation of the cytoskeletal-regulating GTPase Rho (Cappuccio et al., 2010). Though the direct mechanism was not defined, it was shown that Orc3 negatively regulated the activity of Rho, in turn modulating the activity of mGlu4 receptors (Cappuccio et al., 2010). The in-depth analysis of Orc provided the data to support the hypothesis that cell cycle proteins play multifaceted roles and expanded the investigation to other known cell-cycle proteins.

Several lines of evidence have implicated factors involved in mitotic progression in unexpected roles in post-mitotic neurons. One of the most extensively studied is the anaphase promoting complex (APC/C), an ubiquitin-ligase involved in the transition from metaphase to anaphase (reviewed in McLean et al., 2011). Non-cell cycle functions for APC/C in the nervous system include synapse development, survival and metabolic regulation (reviewed in Puram and Bonni, 2011). More recent evidence has examined the mitotic regulator Aurora Kinase A (AurA). The initial observation that NMDA receptor activation led to AurA activation resulted in the identification of novel substrates involved in mRNA stability and translation (Huang et al., 2002; Sarkissian et al., 2004). Additional roles for AurA in neurite extension and post-mitotic cytoskeletal dynamics have also been described (Khazaei and Puschel, 2009; Mori et al., 2009; Takitoh et al., 2012). APC and AurA represent components of the mitotic machinery that have been extensively characterized in non-classical processes in mature neuronal populations.

There are many studies supporting the idea that factors involved in G_1/S progression can effect non-cell cycle processes in post-mitotic neurons (Glickstein et al., 2007; Joseph et al., 2003; Nguyen et al., 2006a). The role of neuronal Cyclin E has perhaps the most complete characterization. Based upon the initial observation that residual Cyclin E expression remains in the synapses of post-mitotic neurons, Cyclin E was shown to have a function in regulating synaptic physiology (Miyajima et al., 1995; Odajima et al., 2011). A differential proteomic screen identified a Cyclin E-CDK5 interaction in post-mitotic neurons than was not present in embryonic neural precursors (Odajima et al., 2011). CDK5 is an atypical CDK which binds the non-Cyclin effector protein p35 (Tsai et al., 1994). CDK5 and p35 have been shown to be essential for many facets of nervous system function including development, apoptosis and synaptic activity (Chae et al., 1997; Su et al., 2012; Tian et al., 2009). Functional analysis revealed a reduced number of synapses and dendritic spines in Cyclin E null animals, which could be rescued by modulation of CDK5 activity (Odajima et al., 2011). These molecular and biochemical links between CDK5/Cyclin E were accompanied by physiological defects in learning resulting from disruption of synaptic plasticity (Odajima et al., 2011). The study of Cyclin E and its role in synaptic function is one of the most comprehensive works detailing the importance of a molecular interaction between a cell cycle regulatory factor and physiological processes in the nervous system. **An important aspect of this thesis is to translate observed molecular interactions by the Rb/E2f pathway into physiological relevant processes.**

2.0 The Retinoblastoma Protein – An Overview

From its initial identification in 1983, the retinoblastoma protein has not only served to expand our knowledge of its specific role in cancer progression but also as a prototype to

understand how other inherited mutant alleles contribute to specific malignancies. The notion that *RB1* mutation can lead to cancer expanded the oncology field by implicating viral-oncoproteins in Rb inactivation (reviewed in DeCaprio, 2009), identifying the Rb-pathway as commonly deregulated in most cancers (reviewed in Burkhart and Sage, 2008), and verifying Knudson's two-hit hypothesis for cancer initiation (Knudson, 1971). Additionally, the discovery of the retinoblastoma protein led to the characterization of the pathway governing the transition from G_1 to S phase of the cell cycle (reviewed in Burkhart and Sage, 2008). The discovery that Rb participates in cell cycle regulation helped to reinforce the notion that cancers arise due to uncontrolled cellular proliferation (discussed in Nowell, 1978). The identification of Rb led to the discovery of two other members of the pocket protein family, p107 and p130, as well as the E2f family of transcription factors (E2f1-8). **The physiological contribution of *RB1* to cancer progression and nervous system function will be reviewed. Specific focus will be placed upon unique roles for Rb in gene regulation and how this imparts cell-type specific functions for the Rb/E2f pathway.**

2.1 The Retinoblastoma Protein in Tumour Suppression

2.1.1 Retinoblastoma and RB1 Discovery

Retinoblastoma is a pediatric malignancy that affects approximately 1 in 15000 children (reviewed in Dimaras et al., 2012). An initial hypothesis by Alfred Knudson, using retinoblastoma as a model cancer, challenged the current dogma that cancers arise from a large number of mutations by proposing that this disease could arise from as few as two (Knudson, 1971). Using statistical techniques applied to a cohort of 48 retinoblastoma

patients, he proposed that 1) in familial forms of the disease, a single germline mutation is present followed by a second somatic mutational event and 2) in sporadic cases, both mutational events occur in somatic cells (Knudson, 1971). This was reinforced with the predominance of unilateral lesions in sporadic cases, where bi- and tri-lateral disease was only observed in dominantly inherited familial form of the disease. Sporadic cases also have a later onset which supports the notion that two mutations need to be acquired before initiation of disease (Knudson, 1971). Knudson's work relied on statistical models to provide insight into disease and still required significantly more in-depth experimentation to validate.

Knudson's notion came to "molecular fruition" a decade later with the identification and cloning of a locus containing the retinoblastoma susceptibility gene product (Benedict et al., 1983; Cavenee et al., 1983; Cavenee et al., 1985; Friend et al., 1986; Godbout et al., 1983). The retinoblastoma susceptibility locus was mapped to the long-arm of chromosome 13 (13q14.2), spanned 183kb and contained 27 exons resulting in an 100kDa protein (Benedict et al., 1983; Cavenee et al., 1983; Cavenee et al., 1985; Godbout et al., 1983; Hong et al., 1989). Further analysis identified Rb as a nuclear phospho-protein with DNA binding capacity (Lee et al., 1987b; Lees et al., 1991; Lin et al., 1991) and its mRNA reduced (or absent) in retinoblastoma (Lee et al., 1987a). Subsequently, it was discovered that germline mutation in a single allele of *RB1* promoted a loss of heterozygousity (Lee et al., 1987a). This discovery confirmed Knudson's two hit hypothesis and provided molecular evidence that retinoblastoma arises from biallelic inactivation of *RB1*. Additional physiological and mechanistic insight into pRb function was again put on hold for a decade

until the emergence of transgenic *murine* models (Brinster et al., 1981; Brinster and Palmiter, 1984).

The ability to remove specific genes from the mouse genome held the potential to easily model retinoblastoma in a genetically tractable system. The race to produce the first Rb null mouse was met with great disappointment. Three parallel studies all reported that germline heterozygous animals for *RB1* did not develop retinoblastoma (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). These animals survived and, aside from low-penetrance pituitary tumour formation reported in (Jacks et al., 1992), did not have any overt phenotype. Animals null for both *RB1* alleles exhibited early embryonic lethality due to massive apoptosis which was particularly elevated in the CNS (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The absence of tumor formation in this initial mouse model presented two important issues: 1) The progression of retinoblastoma, perhaps owing itself to compensation by other members of the pocket protein family, differs in mice and humans and 2) The need for a preclinical model to study retinoblastoma *in vivo.*

The answer to both of these aforementioned issues was resolved with the combination of genetic inactivation of *RB1* along with other pocket protein family members. The first evidence for pocket protein compensation was provided by combined germline inactivation and *RB1* and *p107*. Chimeric animals with Rb/p107 null cells presented with retinoblastoma, providing evidence that p107 can act as a tumour suppressor and was indeed the roadblock to tumour formation in single *RB1* mutants (Robanus-Maandag et al., 1998). The introduction of cell-type specific conditional knock-out models provided further evidence for the compensatory role of pocket proteins in mouse retinoblastoma. Three independent studies utilizing different conditional retinal deletion models (Pax6, Nestin and

Chx10) reported high-penetrance retinoblastoma formation upon deletion of Rb in conjunction with either p107 or p130 (Chen et al., 2004; MacPherson et al., 2004; Zhang et al., 2004b). This was the first work to implicate p130 as a tumour suppressor in the absence of Rb. These studies all implicated retinal progenitor cells in the pathogenesis of retinoblastoma, as highlighted by the presence of progenitor markers within tumours (Chen et al., 2004; MacPherson et al., 2004). These aforementioned studies are at odds with regards to the contribution of lineage-specificity in the pathogenesis of disease (Ajioka et al., 2007; Chen et al., 2004; Xu et al., 2009; Zhang et al., 2004a). It is unknown whether increased progenitor proliferation, cell cycle exit-delays or cell-cycle re-entry and de-differentiation contribute to the disease. The two latter hypothesis have been supported by additional studies as more differentiated cell-types were shown to be the origin of retinoblastoma in other mouse models (McEvoy et al., 2011; Vooijs et al., 2002; Xu et al., 2009). Though much evidence has speculated at the cancer initiating population, the origin of retinoblastoma is still controversial (discussed in Dyer and Bremner, 2005).

It is still unknown why retinal cell types display an increased susceptibility to tumour formation following *RB1* mutation. Owing to the advancements in diagnostic and treatment protocols for retinoblastoma, most patients are successfully treated (Broaddus et al., 2009; Chan et al., 2005). The elevated number of retinoblastoma-survivors led to the observation that these children exhibit an increased susceptibility to a number of other specific malignancies.

2.1.2 RB1 Cancer Syndrome

Retinoblastoma patients exhibit an extremely positive outcome when disease is detected early (Broaddus et al., 2009). The successful treatment of retinoblastoma is achieved with a cocktail of radiation and aggressive chemotherapeutics such as alkylating agents (nitrogen mustards), and DNA damage inducers (topoisomerase inhibitors and anthracyclines) (reviewed in Chan et al., 2005). Aggressive treatment regimes, as with retinoblastoma, often come at the sacrifice of further malignancy resulting from the toxic therapeutics (reviewed in Moppett et al., 2001). An initial observation in retinoblastoma was the presentation of very specific tumour-types in previously treated patients (Gurney et al., 1995). The specificity of tissue and tumour type could not be solely attributed to post-treatment malignancy or recurrence of metastatic disease (Gurney et al., 1995). These secondary malignancies were observed most commonly in familial forms of retinoblastoma, suggesting a role for germline *RB1* mutation (Gurney et al., 1995). The most predominant and first reported was osteosarcoma, which occurs at a staggering 500 fold increased incidence (Gurney et al., 1995).

The high-penetrance of osteosarcoma in familial retinoblastoma patients poses two interesting questions: Why do mesenchymal-derived osteoblasts display an increased susceptibility to *RB1* mutation? Why do mesenchymal-derived osteoblasts display a more latent disease onset compared to retinal cells? Similar to retinoblastoma, the cell of origin in osteosarcoma is not yet known (discussed in Liu et al., 2011; Mohseny and Hogendoorn, 2011). It is hypothesized that mesenchymal stem cells or osteoprogenitor cells are the initiating population in the disease (Liu et al., 2011). As opposed to a more one-dimensional role in cell cycle progression and exit, the role of Rb in mesenchymal cells is more diverse.

Seminal studies in adipose tissue, another mesenchymally derived cell-type, showed that Rb plays a role in governing the fate choice between brown and white adipose tissue (Hansen et al., 2004; Scime et al., 2005). The later study implicated direct Rb/E2f regulation of PGC-1α, a transcription factor involved in energy metabolism, in governing this fate switch in adult progenitors (Scime et al., 2005). This was an important finding, as it was one of the first studies providing direct mechanistic and *in vivo* evidence that Rb played a role in regulating a non-cell cycle factor. This complemented an initial finding that Rb bound and positively regulated the transcriptional activity of the osteogenic transcription factor Runx2 (Thomas et al., 2001). These two independent findings were later reconciled in a study revealing that Rb plays a more upstream role in mesenchymal differentiation (Calo et al., 2010). In an *in vivo* model of osteosarcoma (Rb^{flox}, $p53^{flox}$), driven in uncommitted mesenchymal cells (Prx1-Cre), Rb was shown to govern the fate choice between brown fat and bone (Calo et al., 2010). Through interactions with the non-cell cycle related transcription factors, PPARγ and Runx2, Rb-loss was paradoxically shown to increase the adipose-fate of uncommitted cells (Calo et al., 2010). These findings led to the hypothesis that *RB1* mutant osteosarcomas may arise due to the de-differentiation of more committed osteoblast cells (Calo et al., 2010). This hypothesis has recently been re-enforced with the finding that adipocyte precursors may be the initiating population in rhabdomyosarcoma, a rare muscle derived tumour (Hatley et al., 2012). This supports the hypothesis that de- or trans-differentiation may be causative factors in mesenchymal-derived tumours. These studies provided evidence that the role of *RB1* mutation in cancer may extend beyond cell cycle regulation.

The examination of non-classical and classical roles for Rb in cancer progression has expanded our knowledge of the pathogenesis of other cancers in the *RB1* spectrum including melanoma, lung and bladder tumors (reviewed in Moll et al., 2012; Sage, 2012). It is important to understand the tissue and cell-type specific differences in Rb and E2f function in order to fully appreciate their roles in disease.

2.2 The Retinoblastoma Protein in Neural Precursors

Since the implementation of transgenic technologies, much emphasis has been placed on the role of the retinoblastoma protein in the CNS. The specific emphasis on CNS function is largely due to the CNS-origin of the retina; however, the wide-spread nervous system disruption in the germline *RB1* deletion animals piqued the interest of the research community (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). These animals exhibited CNS-specific defects that included apparent increases in apoptosis, differentiation defects and overall structural abnormalities (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The presence of these defects reinforced the notion that Rb plays CNS- roles and understanding these specific functions will help shed light on its tumour suppressive function.

The notion that Rb loss disrupted the neural differentiation program in the CNS was an intriguing finding from initial reports. Similar to data obtained in mesenchymal cell lineages, Rb may act outside the cell cycle to influence tumourigenesis in the developing retina. Initial mouse models held the promise of identifying the cell of origin in retinoblastoma. This initial promise faded with multiple studies each reporting a unique cell-type of origin for retinoblastoma (Ajioka et al., 2007; Chen et al., 2004; Xu et al., 2009;

Zhang et al., 2004a). The recurring themes within these studies were that specific sub-types were altered by pRb loss and that they all displayed delayed cell cycle exit. The model of delayed cell cycle exit suggests that several ectopic proliferation events in a committed neural precursor may lead to the increase cell number observed in retinoblastoma. In fact, in the retina and other CNS precursor populations, no pronounced increases in proliferation were observed in more rapidly dividing populations (Chen et al., 2004; Ferguson et al., 2002; MacPherson et al., 2003). This provided evidence for common function for Rb in the CNS, but did not provide an explanation as to why retinoblastoma arises following *RB1* mutation and not other pediatric CNS tumours such as medulloblastoma or neuroblastoma. These differences in tumour-type may extend beyond cell cycle regulation.

The most well described non-cell cycle role for Rb in central nervous system development has been its role in forebrain development. The conditional ablation of Rb in the CNS provided interesting contrasts to the phenotypes observed in the germline mutant animals (Ferguson et al., 2002; MacPherson et al., 2003). As opposed to the massive CNS defects, the two reports of conditional ablation in the forebrain reported more modest phenotypes (Ferguson et al., 2002; MacPherson et al., 2003). The conditional mutant displayed near normal levels of immature neurons; however, more in depth analysis revealed defects in cortical lamination (Ferguson et al., 2002). Conditional removal of Rb in the forebrain is therefore compatible with neuronal differentiation and differentiation can occur without proper withdrawal from the cell cycle (Ferguson et al., 2002). Defects in lamination in the dorsal cortex were postulated to occur as a result of the death of Cajal-Retzius neurons, a Reelin-expressing neuronal subtype essential for proper radial migration (Ferguson et al., 2005). More intriguing was the apparent defect in tangential interneuron

migration, a population which was not significantly confounded by ectopic mitosis (Ferguson et al., 2005). This cell population allowed for the dissection of Rb-regulated neuronal migration, in the absence of potential proliferation defects (Ferguson et al., 2005). To this end, it was shown, using an elegant series of transplantation experiments, that disrupted interneuron migration was cell-autonomous (Ferguson et al., 2005). Subsequent studies implicated E2f3 in the regulation of neuronal migration, though the specific gene targets were not identified (McClellan et al., 2007). Microarray analysis revealed several putative factors including ApoE, Sema3d, Twist and Neogenin (McClellan et al., 2007). **The examination of the direct regulation of the non-cell cycle factor, neogenin, in mediating the aberrant migration in the absence of Rb is a focus of this thesis.** Similar studies in the retina implicated Rb-E2f3a interactions in cell cycle independent differentiation of starburst amacrine cells, a specific retinal interneuron population (Chen et al., 2007). Specific gene-targets were not identified; however, this would present another interesting system to study cell-cycle independent Rb functions.

2.3 The Retinoblastoma Protein: Beyond Cell Cycle Regulation in the Nervous System

An unexpected, but nonetheless exciting, observation from the germline *RB1* mutant transgenic animals was the massive apoptosis observed in the CNS. Though embryos did exhibit increased levels of cell death in other tissues, it was particularly apparent in the CNS (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Though later it would be attributed to a non-autonomous placental defect (de Bruin et al., 2003; Wu et al., 2003), this observation sparked the interest and established Rb as a *bona fide* apoptotic regulator (reviewed in Polager and Ginsberg, 2009). These studies also complemented observations

that ectopic cell cycle events were a common occurrence in neurodegenerative diseases (reviewed in Herrup and Yang, 2007).

The perspective that cell cycle entry presented a method of cell death in the CNS was again another piece of evidence that Rb function and loss is tolerated differently in a cell-type specific manner. As opposed to retinal neurons, which tolerate and even proliferate in a terminally differentiated state (Ajioka et al., 2007), this was not the case for cortical and other neuronal types (Liu et al., 2008; Varvel et al., 2008; Verdaguer et al., 2002). *In vitro* analysis of cortical neurons treated with a variety of apoptosis-inducing agents revealed that Rb phosphorylation was as an early-event prior to cell death and that blocking E2f-mediated transcription attenuated the cell death response (Park et al., 2000). In contrast, studies in other neuronal systems using broad-scale pocket protein inhibition by infection with viral oncoproteins did not report a cell death response (Slack et al., 1998). Short-term inactivation of Rb with siRNA did not have any effect on steady-state neuronal survival (Liu et al., 2005). Studies using genotoxic agents implicate the Rb/E2f pathway in neurodegeneration; however, analyses of phenotypes associated specifically with Rb removal in post-mitotic neurons have not been performed. The compensatory role of other pocket protein family members may account for variation in phenotypes observed in quiescent neurons. **Identifying the physiological requirement and gene targets for Rb in terminally-differentiated quiescent cortical neurons is a focus of this thesis.** It is pivotal to examine the short and long-term effects of acute Rb deletion in post-mitotic cortical neurons.

Few studies have shown a non-cell cycle role for the pRb in post-mitotic neuronal populations. This may be due to the constitutive nature of Rb repression in quiescent cell types, as one would imagine that neuronal-specific factors that require modulation in

expression would not be ideal candidates for regulation by E2fs in a G_0-state. In horizontal cells, the loss of Rb was shown to alter dendritic arborisation in a cell autonomous manner (Martins et al., 2011). Though it occurred autonomously, it was not detailed whether this may be a non-specific cell cycle related defect or direct regulation of a specific target gene(s) (Martins et al., 2011). It is imperative that specific Rb regulated gene targets are identified in order to fully appreciate its cell type specific role. Analysis of acute Rb deletion in post-mitotic cortical neurons will allow for the identification of potential novel cell cycle independent targets.

3.0 Mechanisms of Rb-mediated Gene Repression – An Overview

In summary, Rb impedes cell cycle progression during G_1 by binding and inhibiting the transcriptional activity of E2fs. The phosphorylation of Rb by CDK-Cyclin complexes prevents its interaction with E2f and allows for the transcription of genes involved in cell cycle progression. In order to create potential therapeutics and determine how Rb-related de-regulation can lead to tumour formation, it is essential to identify how the structure of Rb translates into its functional role.

3.1 Structure and Function of Rb

A puzzling notion from the initial biochemical observations that Rb bound to DNA and the E2f transcription factors, was the absence of any recognizable DNA-binding or protein-interaction domain within its sequences (Benedict et al., 1983; Cavenee et al., 1983; Cavenee et al., 1985; Friend et al., 1986; Godbout et al., 1983). Analysis of crystal structures revealed two stereotypic 'Cyclin folds' within Rb but most domain interactions/structures have been deletion mapped (Figure 1.3) (reviewed in Dick, 2007).

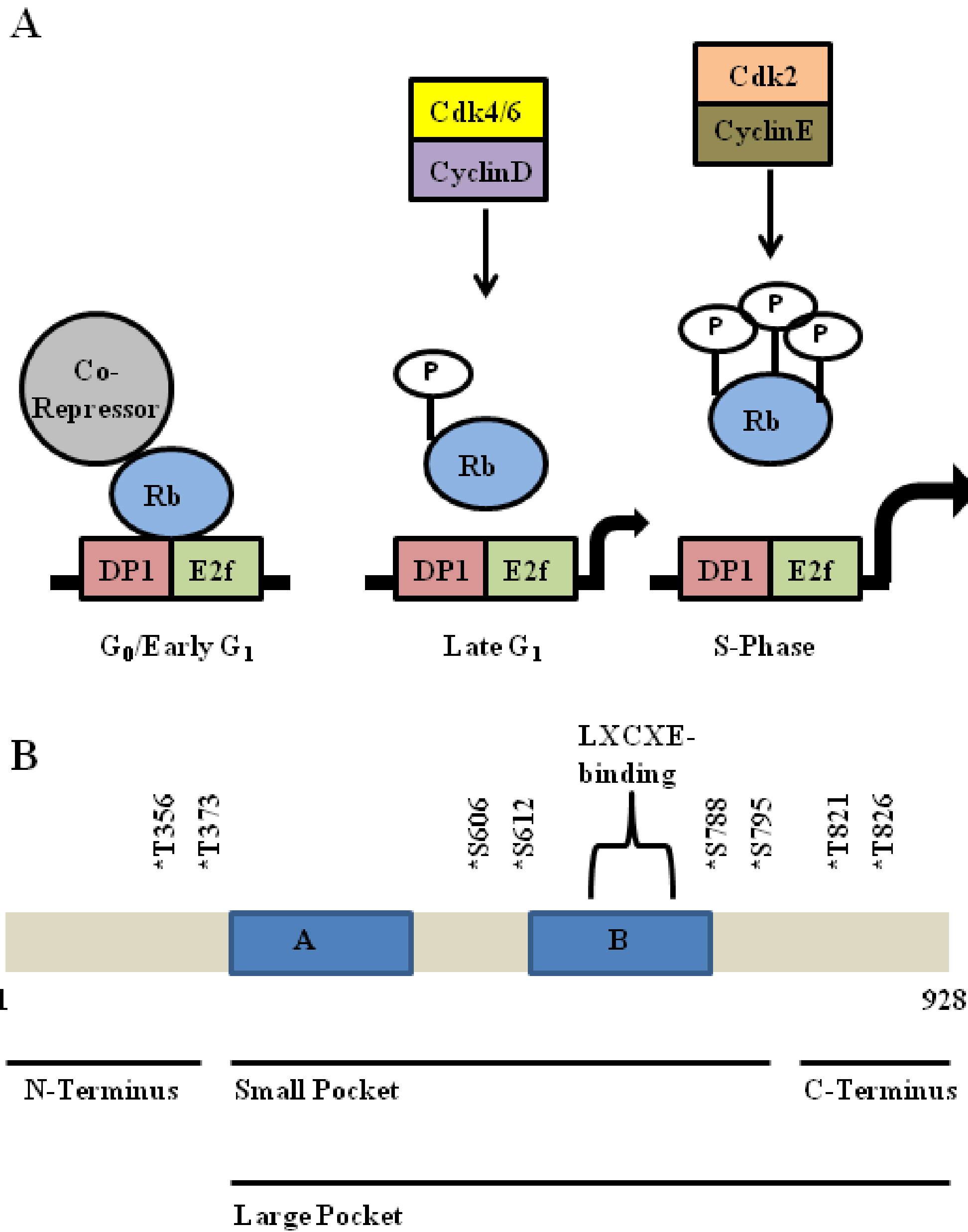

Figure 1.3 Structure and function of the retinoblastoma protein. A) During cell cycle arrest (G0/G1), Rb is bound to E2f and repressive co-factors preventing expression of S-phase genes. During Late G1, Rb is phosphorylated by Cdk4/6/CyclinD complexes alleviating repression of E2f. Further phosphorylation of Rb by Cdk2/CyclinE drives the cell into S-phase B) Structure of Rb. Important phosphorylation sites are denoted with *.

Figure adapted from (Gordon and Du, 2011), (Dick, 2007)

The analysis of tumour-derived mutations did not provide the mechanistic insight into Rb function as it has with other tumour suppressors and oncogenes (Ras, Ptch, Smo) (Dong et al., 2000; Finkel et al., 1984; Xie et al., 1998). Rb has been grossly divided into four domains: The small and large pocket domains and the C- and N-termini. The small pocket domain was characterized as the minimal region required to bind viral oncoproteins (reviewed in DeCaprio, 2009). The large pocket was later defined as the region required to mediate the growth suppressive activity of Rb (Qin et al., 1992). The C and N terminal regions of Rb carry-out less well defined functions. In general, both are involved in protein-protein interactions that vary from non-classical E2f1 binding (Dick and Dyson, 2003), alternative growth arrest by p27 binding (Ji et al., 2004) and chromatin remodeling (reviewed in Talluri and Dick, 2012). Chromatin remodeling is largely thought to be mediated by the LXCXE-binding domain in the Rb protein (Talluri and Dick, 2012). The LXCXE-binding domain is located within the small pocket and was identified as a binding site for viral oncoproteins (reviewed in Dick, 2007). The role that LXCXE-binding plays in regulating protein-protein interactions and Rb function will be described in detail in subsequent sections.

Crystal structures of Rb in inactive and active states have been of great value to our understanding of Rb's dynamic structure in different activation states. Insight into Rb function from crystallography lagged behind the initial identification of the Cyclin-binding regions and deletion-based experiments. The analysis of an Rb-E2f1-DP1 structure revealed that phosphorylation of Rb at threonines 821 and 826 destabilized the complex, allowing for E2f-activation (Rubin et al., 2005). Subsequent analysis revealed that multiple phosphorylation events are able to induce conformational changes that inhibit binding to the

E2f transactivation domain (Burke et al., 2010). The interaction between PP1c, a protein phosphatase, and Rb was confirmed to occur in the C-terminal and overlaps with a CDK docking site (Hirschi et al., 2010). This provided evidence that kinase and phosphatase activities may be in competition to sculpt the activation state of Rb. Recent studies examining the structure of phosphorylated Rb were the first to provide structural-evidence to support observed deletion studies. Phosphorylation of serine 309 was shown to create a 'flexible pocket loop' which in turn blocked the E2f-transactivation domain, whereas threonine 373 phosphorylation induced a conformational change that associates the pocket and N-terminus masking LXCXE binding (Burke et al., 2012). These structural experiments have focused on phosphorylation induced changes in Rb and have been imperative in our knowledge regarding the cyclical nature of Rb activity. Structural changes in phospho-Rb have helped us understand the initiating events in E2f activation.

3.2 E2f Transcription Factors in Rb Function

The most well characterized protein-protein interaction that Rb elicits is that of E2f. It is the fundamental interaction between these two proteins which shapes their output and physiological outcomes. As with Rb, E2f's function in a tissue and cell type specific manner (reviewed in Chen et al., 2009). A wealth of literature is available on E2f function and this will merely serve as an introduction to their function in respect to Rb. For more in depth discussion of E2fs please consult the following recent reviews (Chen et al., 2009; Polager and Ginsberg, 2008; Wong et al., 2011).

Fundamentally E2f's can be divided into two specific categories: activators (E2f1-3a) and repressors (E2f3b-E2f8). This broad classification is based upon known interaction

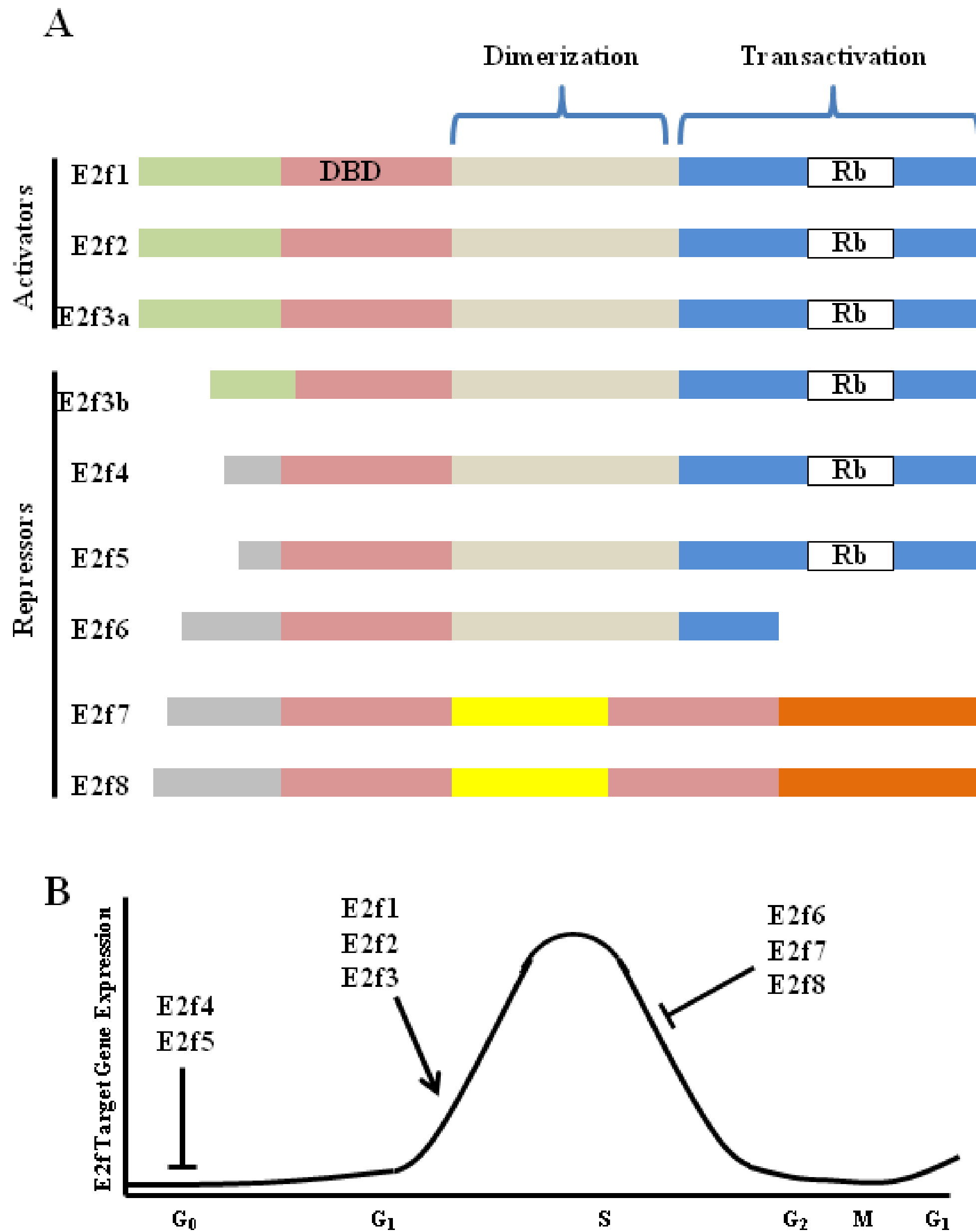

Figure 1.4 Structure and expression of mammalian E2fs. A) Structural schematics of E2fs. Common colours denote regions of significant homology. DBD indicates the DNA binding domain and Rb indicates the region bound by pocket proteins. B) Graphical representation of E2f-target gene expression versus cell cycle phase and corresponding E2f activity (repression/activation)

Figure adapted from (Chen et al., 2009)

data between pocket proteins throughout the cell cycle and their overall E2f-expression landscape (Figure 1.4) (Takahashi et al., 2000) and reviewed in Wong et al., 2011). E2f1-5 interact with DNA as a heterodimer along with DP1-3 (Girling et al., 1993; Helin et al., 1993). E2f6-8 lack a trans-activation domain and are incapable of eliciting an activation response (Chen et al., 2009). E2f's 6-8 also lack a pocket protein binding domain and act as constitutive repressors independently of pocket proteins. Non-Rb interacting E2fs will not be discussed; although, it is interesting to note that their expression is elevated in the absence of Rb. In the absence of all three pocket proteins, cells are able to initiate a G_1 arrest (Wirt et al., 2010). This lends itself to the hypothesis that atypical repressive E2f's may function to repress E2f-sites normally bound by Rb/E2f complexes in the absence of specific pocket proteins (discussed in Wirt et al., 2010). Data supplementary to this thesis and additional recent studies have provided physiological and mechanistic support for this notion (Aksoy et al., 2012; Ghanem et al., 2012).

The activator nomenclature for E2fs is dependent on their ability to elicit transcriptional activation but, it does not preclude an inability to repress transcription in the presence of the appropriate pocket protein. Indeed, all three classical E2f activators exist in repressive complexes bound to Rb at intervals during the cell cycle (Chong et al., 2009; Johnson et al., 1994). Both indirect and direct interactions between Rb and activator E2fs have dominated the literature regarding Rb/E2f function. The removal of E2f1 and/or E2f3 in conjunction with Rb has been shown to rescue phenotypic disturbances including tumor formation (Bourgo et al., 2011; Hurst et al., 2008; Sangwan et al., 2012), apoptosis (Chen et al., 2007; Wenzel et al., 2007) and non-cell cycle effects (McClellan et al., 2007; Saenz

Robles et al., 2011). These interactions are straight-forward and rely on the initial model proposed in which Rb and E2f act in a binary fashion to regulate gene-expression events.

E2f4 is the only classical repressor E2f that has been reported to interact with Rb (Nakajima et al., 2007). The repressor term refers to the idea that during normal physiological conditions, repressor-E2fs exist in a complex with a pocket protein and not in a free transcriptionally active state (reviewed in Blais and Dynlacht, 2007). E2f4 mediates its transcriptional repression by participation in DREAM, a large repressive complex capable of recruiting chromatin re-modeling enzymes (Blais and Dynlacht, 2007). Though scenarios in which extra-physiological levels of E2f4 are able to produce an activation response (Lee et al., 2011), there is scarce evidence to suggest this occurs under normal cellular conditions. It is widely accepted that repressor complexes are traded for E2f activators to initiate transcription (Frolov et al., 2001; Tyagi et al., 2007). It is important to note that repressor-E2fs are not a prerequisite for long-term gene repression by Rb/E2f complexes (Chong et al., 2009).

In most cellular contexts, including quiescence entry, E2f4 is thought to interact with p130 and not Rb (Flink et al., 1998; Jori et al., 2005; Puri et al., 1997). The interaction between Rb and E2f4 has been tenuous in terms of its observed biochemical interaction and a subsequent physiological outcome, as it is largely based upon genetic interactions. Initial evidence revealed that removal of E2f4, in sporadic tumours from germline *RB1* heterozygous mice, showed a decrease in tumor formation (Lee et al., 2002). The mechanism for this tumour suppression was not shown, but it was suggested to occur by re-arrangement of pocket protein-E2f complexes. In the absence of E2f4, the liberated p107 and p130 would be able to repress activator E2fs, thus compensating more efficiently for the

removal of Rb (Lee et al., 2002). In a model of Myc-induced lymphoma, E2f4 was shown to delay disease onset suggesting a potential function of E2f4 independent of Rb (Rempel et al., 2009). This is re-enforced by evidence during normal hematopoetic system development, as Rb and E2f4 genetically interact yet, this interaction is suggested to occur due to independent cell-intrinsic factors not direct regulatory functions (Zhang et al., 2010). It is interesting to note that E2f4 is one of the few E2fs in which expression is not de-regulated in the absence of Rb (Blais et al., 2007; Bourgo et al., 2011; Wirt et al., 2010). The few studies displaying physical interaction between Rb and E2f4 have relied upon artificial expression systems and may not reflect normal biological function (Nakajima et al., 2007; Paramio et al., 2000). The precise role that Rb and E2f4 play during gene regulation and physiological events is unclear but likely occurs in an in-direct manner.

The interactions between Rb and E2f are relatively simplistic. Expansion on the initial dogma of Rb and E2f function has lead to more comprehensive mechanistic insight into their regulation of gene transcription. Rb does not act to repress gene expression independently nor does E2f activate transcription without the aid of accessory factors. These events are crucial in Rb/E2f function.

3.3 Influence of Rb on Chromatin Remodeling

Rb and E2f function in a complex to facilitate regulation of antagonistic transcriptional events. The activation of gene transcription in the absence of pocket proteins requires a coordinated effort, ultimately resulting in the recruitment of RNA polymerase to gene promoters. Alternatively, in a repressive state, promoters need to be in a conformation that impedes the recruitment of RNA polymerase. Rb and E2f have expansive roles in

regulating the complex processes of transcriptional activation and repression. **A focus of this thesis is the examination of the changes in chromatin activation-state in the absence of Rb and the mechanisms which may governs theses changes.**

3.3.1 E2f Activation

The activation of gene transcription requires a conformational change in chromatin, allowing access to the gene promoter by the necessary core transcriptional regulatory enzymes such as RNA polymerase II (reviewed in Razin et al., 2011). Transcriptional-activators need to, by direct or in-direct means, regulate these changes in chromatin architecture. The mechanistic roles for E2fs in transcriptional regulation have lagged behind that of pocket proteins though, several key studies have identified potential mechanisms for E2f-activation.

An initial observation upon E2f-dependent activation of cell-cycle genes was a concurrent increase in acetylation of histones 3 and 4 (Nicolas et al., 2003; Takahashi et al., 2000). Several histone acetyltransferases have been putatively implicated in this phenomena (Ait-Si-Ali et al., 2000; Morris et al., 2000; Taubert et al., 2004; Trouche et al., 1996), none of which have yet to be validated in an *in vivo* or pathological setting. An emerging mechanism for chromatin remodeling by E2f is the recruitment of the MLL complex to E2f responsive promoters. MLL is able to activate gene transcription by depositing the H3K4me3 mark. Several independent studies have shown physical interactions between E2f and MLL, as well as alteration in activation state, histone acetylation patterns and ultimately cell cycle progression (Nightingale et al., 2007; Revenko et al., 2010; Tyagi et al., 2007). Additionally, E2f's have also been shown to interact with TATA-binding protein, a factor in

the basal TATA box mediated transcriptional activation machinery (Pearson and Greenblatt, 1997; Peng et al., 2007). Studies examining E2f-activation suggest a heterogeneous response to initiate transcription that may contribute to the context-specific function of E2fs and the impact the repressive activity of Rb.

3.3.2 Rb-mediated Repression

The repressive activity of Rb and E2fs has been on on-going facet of Rb/E2f mechanistic investigation. Rb and pocket protein family members have been shown to recruit enzymes that deacetylate, methylate and ultimately remodel chromatin to alter its activation state (reviewed in Blais and Dynlacht, 2007). This introduction will focus on interactions that have a more in-depth physiological role.

An initial observation during muscle differentiation was an increase in H3K9me3 and its enzyme Suv39h1 at S-phase genes, entering into a terminally differentiated state (Ait-Si-Ali et al., 2004). This mark was not present in cycling cells, suggesting a unique quiescence-based silencing system (Ait-Si-Ali et al., 2004). This provided a physiological complement for the molecular finding that Rb bound HP1 (a Suv39h1 co-factor) and methylated histone 3 (Nielsen et al., 2001). In the nervous system, increased HP1 occupancy at E2f-responsive promoters was correlated with terminal differentiation (Panteleeva et al., 2007). Though no direct interaction between HP1-Rb-E2f was shown in this context, it provides evidence that they may act in concert to mediate their overall repressive effects. Rb has subsequently been linked to HP1/Suv39h1 in regulating quiescence in cardiomyocytes (Sdek et al., 2011). Similarly, changes in the Polycomb group mediated H3K27me3 mark at E2f-responsive promoters, has been correlated with the differentiation state, though no

direct-interaction was reported (Blais et al., 2007). A reciprocal de-regulation of Rb-E2f was reported in Polycomb group mutants suggesting a potential for cross-talk between the two pathways (Fasano et al., 2007; Ferres-Marco et al., 2006). Rb-interactions with methylating enzymes established a model whereby E2f responsive genes are silenced at the level of chromatin. A more important event in gene activation may be the transition from the activated to deactivated chromatin state by removal of activating marks.

The most well characterized Rb-mediated chromatin interaction occurs with class I histone deacetylases (HDACs). HDACs act to silence gene transcription by removing acetyl groups from activated histones (reviewed in Thiagalingam et al., 2003). This initial step allows a gene to transition from an activated to silenced state. One of the first interaction partners of Rb characterized was HDAC1 (Ferreira et al., 2001; Lai et al., 1999; Magnaghi-Jaulin et al., 1998; Zhang et al., 2000). The Rb-HDAC complex was first implicated in simple cell cycle dynamics, with this interaction shown to strengthen Rb-E2f mediated repression during G_1 (Ferreira et al., 2001; Lai et al., 1999; Magnaghi-Jaulin et al., 1998; Zhang et al., 2000). This interaction was reinforced in cells entering a quiescence state, with an importance observed during adipocyte (Fajas et al., 2002) and neural (Jori et al., 2005) differentiation. In-direct analysis has also implicated HDAC1 function, by mutation of Rb's LXCXE interaction domain, to other Rb-mediated processes (Dahiya et al., 2000; Ferreira et al., 1998). The LXCXE motif provides a structural component in order to elucidate mechanistic insight into Rb-HDAC1 and other putative chromatin modifying Rb-interacting enzymes.

3.3 LXCXE Interactions – Linking Rb to Chromatin Remodeling

The Rb LXCXE binding motif was of great interest to the cancer research community as it was shown to bind viral oncoproteins during the inactivation of Rb (reviewed in DeCaprio, 2009). This not only provided insight into the pathogenesis of these viral factors but is also a useful tool to apply to other biological contexts. The Rb LXCXE binding motif has been shown to be involved in the interaction with many factors (Table 1.1) (reviewed in Dick, 2007). In this introduction, focus will be placed on interactions with a physiological component, as many initial *in vitro* studies have yet to translate into *in vivo* and more advanced molecular biology systems. **A focus of this thesis is the role LXCXE interactions play in the regulation of gene repression in quiescent cortical neurons.**

Initial studies examining LXCXE-dependent function of Rb focused on its classical role in cell cycle regulation. Indeed in several models, including fibroblasts, mutation of the LXCXE-binding domain impeded Rb-induced G_1 arrest (Chen and Wang, 2000). In contrast, LXCXE-deficient Rb was sufficient to maintain growth arrest in terminally differentiated muscle tissue (Chen and Wang, 2000). In the presence of serum, which myotubes are normally refractive towards, LXCXE mutants re-entered the cell cycle (Chen and Wang, 2000). This suggests a co-ordination between upstream proliferative signals and the ability of Rb to repress gene transcription by LXCXE-dependent interactions. This complements the identification of LXCXE specific phospho-residues on Rb (Knudsen and Wang, 1997), as they may represent a unique means of maintaining myotube quiescence. The importance of LXCXE-associated factors was called into question when another LXCXE-mutant was shown to be dispensable for growth-arrest (Chan et al., 2001). The use of multiple LXCXE mutant variants may contribute to phenotypic variation, as few studies characterized what other non-specific effects these mutations may have on Rb function.

Table 1.1 LXCXE-dependent Rb Interactors

Protein	Function	Reference (s)
AhR	Aryl hydrocarbon receptor	(Ge and Elferink, 1998)
ASK1	Ser/Thr kinase	(Dasgupta et al., 2004)
BRG1	ATP dependent helicase	(Dunaief et al., 1994)
Brm	ATP dependent helicase	(Singh et al., 1995), (Trouche et al., 1997)
CtIP	Transcriptional repressor	(Meloni et al., 1999)
Cap-D3	Chromosome Condensation	(Longworth et al., 2008)
Cyclin D1	Cdk regulatory subunit	(Dowdy et al., 1993)
Cyclin D2	Cdk regulatory subunit	(Ewen et al., 1993), (Kato et al., 1993)
Cyclin D3	Cdk regulatory subunit	(Dowdy et al., 1993), (Ewen et al., 1993), (Kato et al., 1993)
DNA Pol Δ	DNA polymerase	(Krucher et al., 2000)
DNMT1	DNA methyltransferase	(Robertson et al., 2000)
EID1	Unknown	(MacLellan et al., 2000), (Miyake et al., 2000)
ELF1	Transcription factor	(Wang et al., 1993)
HBP1	Transcriptional repressor	(Tevosian et al., 1997), (Lavender et al., 1997)
HDAC1/2	histone deacetylase	(Brehm et al., 1998), (Magnaghi-Jaulin et al., 1998), (Luo et al., 1998), (Lai et al., 1999)
Hec1	Microtubule dynamics	(Zheng et al., 2000)
Hmgb1	Non-histone chromsome structure	(Jiao et al., 2007)
HP1	trimethyl histone H3 binding protein	(Nielsen et al., 2001)
Hsp75	Heat-shock protein	(Chen et al., 1996)
p202	Unknown	(Choubey and Lengyel, 1995)
p204	DNA replication	(Hertel et al., 2000)
Rbbp9	α/β hydrolase	(Cassie et al., 2006), (Woitach et al., 1998)
Rbbp1	Chromatin remodeling	(Defeo-Jones et al., 1991)
Rbbp2	histone demethylase	(Defeo-Jones et al., 1991), (Chicas et al., 2012)
RFC p145	DNA replication	(Pennaneach et al., 2001)
RIZ	methyltransferase	(Steele-Perkins et al., 2001), (Buyse et al., 1995)
Suv39h1	methyltransferase	(Nielsen et al., 2001)
UBF	PolI transcription factor	(Cavanaugh et al., 1995)

Adapted from (Dick, 2007)

The creation of transgenic *murine* models in which an LXCXE-binding mutant was placed into the *RB1* locus has provided more insight into the function of this domain *in vivo*. In accordance with the *in vitro* evidence put forth by the La Thangue group (Chan et al., 2001), initial reports from *in vivo* models showed no broad phenotypic consequences of LXCXE deficiency (Bourgo et al., 2011; Isaac et al., 2006; Talluri et al., 2010). Cells derived from these animals were able to initiate proper growth arrest, and both muscle and retinal neuron cell-types were properly established (Talluri et al., 2010). It is important to note that this model employs a germline homozygous *RB1* LXCXE mutation. Similar to other non-acute models, the homozygous LXCXE mutant displayed compensation by other pocket protein family members when serum was withdrawn (Isaac et al., 2006). It is controversial whether LXCXE-binding factors are required for the establishment or maintenance of terminally differentiated cell types. The use of acute models, even with subtle point mutations, is vital in understanding the specific function of pocket proteins and E2fs due to their compensatory ability.

The LXCXE-binding motif has been controversial in its ability to establish steady-state growth arrest. In contrast, in scenarios of cellular stress, LXCXE has played a more consistent role in regulating E2f transcription. The role of E2f in the regulation of apoptosis occurs by both direct and in-direct mechanisms (reviewed in Polager and Ginsberg, 2008). The involvement of LXCXE now adds the notion of co-factor binding to the role of Rb in these processes. In the presence of DNA-damage it was observed that, through LXCXE-dependent interaction with core DNA replication machinery, Rb is able to promote cell survival (Pennaneach et al., 2001). In the absence of Rb or LXCXE, cells display an increased death response in response to DNA damaging agents (Pennaneach et al., 2001).

This is supported by a recent study using an *in vivo* transgenic approach. In an acute paradigm, LXCXE mutants were shown to have no effect on basal transcription of E2f targets in the liver. After DNA damage, LXCXE mutants displayed increased transcription of E2f targets compared to controls and developed hepatocellular carcinoma (Bourgo et al., 2011). This process was shown to be E2f3 dependent though; the specific LXCXE interacting factor is still unknown. Identification of a novel Rb-LXCXE interaction *in vivo* or validation of previous interacting factor(s) in an *in vivo* context would be extremely informative in modeling tumourigenesis induced by DNA damage.

In the context of initiating tumorigenesis, germline LXCXE mutants where shown to display mitotic defects (Coschi et al., 2010; Isaac et al., 2006). The presence of these defects led to chromosomal instability, a putative initiation event in malignancies (Coschi et al., 2010) and discussed in Bakhoum and Compton, 2012). These defects, in conjunction with p53 deletion, resulted in tumour formation (Coschi et al., 2010). Furthermore, LXCXE mutants accelerated the loss of heterozygosity in p53 (+/-) animals increasing the rate and decreasing the latency of tumour formation (Coschi et al., 2010). Previously identified defects in chromatin condensation in both *Drosophila* and mammalian systems, described an interaction between Rb and the condensin II complex subunt, Cap-D3 (Longworth et al., 2008), which plays an essential role in chromosome assembly and segregation during mitosis (reviewed in Cuylen and Haering, 2011). In human tumour cells, the interaction between condensin and Rb was dependent on the LXCXE-binding domain (Longworth et al., 2008), as its reintroduction to the Rb-deficient Saos-2 cell-line was insufficient to rescue CAP-D3 localization. This provides a mechanistic link between LXCXE and genomic instability yet is has not been determined whether this is the causative event in the tumours

observed in (Coschi et al., 2010). These studies all call into question the fundamental importance of Rb and LXCXE in the maintenance of gene repression versus mitotic progression.

Finally, in the context of cellular senescence Rb has shown an ability to recruit co-factors that differ from normal physiological G_1 arrest (Chicas et al., 2012; Chicas et al., 2010). This hypothesis is supported *in vivo*, as Ras-induced senescence was perturbed in LXCXE mutants (Talluri et al., 2010). No specific interactors were identified; however, since Rb is an essential regulator of senescence, the identification of participating binding partner(s) would be pivotal in understanding the molecular differences underlying quiescence and senescence. Interestingly, previously identified LXCXE interacting protein, Jarid1a (Rbbp2) was shown to regulate senescence by interaction with Rb. It was not confirmed whether this was dependent on LXCXE-binding, though this presents a plausible mechanism (Chicas et al., 2012). The role of LXCXE in stress induced cell-death and senescence serves to highlight the established context-specific roles for Rb in physiologically relevant processes.

4.0 Conclusion

A resounding theme in the study of cell cycle regulation is the notion that these factors play very cell-specific and often non-classical roles. The retinoblastoma protein is no exception. Clinically, *RB1* mutation and disruption of the Rb/E2f pathway results in a mosaic of different tumor types contingent upon latency and cooperating mutation (reviewed in Burkhart and Sage, 2008). In the context of basic developmental events, Rb function is just as diverse. Understanding the cell and tissue specific gene targets and

repressive mechanisms employed by Rb is paramount in our treatment of *RB1*-mutant and Rb/E2f dysfunctional diseases. **This thesis seeks to identify neural cell-type specific mechanisms employed by Rb using the *murine* cortex as a model**. The cortex can be divided grossly into dividing neural precursors and quiescent cortical neurons. The dependence on Rb for tangential migration, presents an excellent physiological paradigm to assess specific Rb-targets in neural precursor cells. Examining Rb function in cortical neurons will allow us to shed light on the controversial role for Rb in this quiescent population. **Our goal is to elucidate overlapping and unique roles within different populations of cells within the mammalian cortex in order to better understand the diversity of Rb function.**

5.0 Statement of Objectives

The physiological roles of Rb and E2fs in the regulation of cell cycle and non-cell cycle processes is well established (reviewed in Burkhart and Sage, 2008; McClellan and Slack, 2007). A resounding question with regard to Rb/E2f function is how they are mediating these differential effects. The nervous system serves as an excellent system to study the differential roles for Rb in an *in vivo* context. In cycling precursors, Rb has been shown to regulate tangential migration in a cell-autonomous manner (Ferguson et al., 2005; McClellan et al., 2007). This provides a scenario to dissect cell cycle-independent mechanisms of Rb function. In contrast, the role of Rb in post-mitotic neurons is controversial (Liu et al., 2005; Park et al., 2000). Thus the goals of the present studies are as follows:

1) To examine the direct-regulation of the non-cell cycle related receptor, neogenin, by Rb/E2f and the role it may play in the observed migration defects in Rb-deficient neural precursors (Chapter 2).

2) To define the role of Rb in quiescent cortical neurons by examining the consequences of acute Rb removal on both a phenotypic and gene regulation level (Chapter 3).

From the above conclusion,

3) To examine the involvement of chromatin remodeling and the LXCXE-binding motif in regulation of Rb-mediated cell cycle gene repression and survival in cortical neurons (Chapter 4).

5.1 Research Objective 1

The presence of a cell-autonomous migratory deficiency, in the absence of cell-cycle exit defects, during ventral forebrain development provides a unique non-cell cycle phenotype upon Rb deletion (McClellan et al., 2007). Tangential migration provides an excellent model to assess specific non-cell cycle gene regulation by Rb in a physiologically relevant context. The identification of several putative migratory factors by microarray laid the groundwork to answer these questions (McClellan et al., 2007).

Hypothesis: Rb directly regulates the expression of neogenin which, in turn, impedes tangential migration of developing interneurons.

In this study, we identify neogenin as a direct Rb/E2f target in forebrain neural precursor cells. We display, by chromatin immunoprecipitation, that E2f3 specifically binds

the 5' neogenin promoter. Luciferase assays confirm that the region identified by ChIP is responsive to E2f3 and Rb. To assess the contribution of neogenin overexpression to the observed phenotype in the absence of Rb, we performed *in vitro* migration and adhesion assays. We observe that Rb-deficient neural precursors display a defective migratory response to the neogenin ligand, netrin. In addition, Rb-deficient precursors also have an increased adhesive capacity to netrin, suggesting a mechanism that may impede their migration. Finally, we show by *ex utero* electroporation and slice culture, that over-expressing neogenin has the capacity to limit migration from the ganglionic eminence. *This finding represents a novel physiological requirement for Rb in the regulation of the non-cell cycle factor, neogenin, during tangential migration. This presents a unique target in neural precursors and presents the possibility that Rb may control other facets of tumourigenesis, such as metastasis, along with proliferation.*

5.2 Research Objective 2

Having identified a direct non-cell cycle Rb target gene in neogenin, we next sought to examine whether Rb/E2f plays a similar role in regulating non-cell cycle gene targets in a quiescent neuronal cell population. The role of Rb in post-mitotic cortical neurons has been controversial (Liu et al., 2005; Park et al., 2000). To alleviate potential confounds by pocket protein compensation; we addressed this question by employing both acute *in vitro* and *in vivo* techniques.

Hypothesis: Rb plays a specific role in cell cycle regulation in quiescent cortical neurons. The maintenance of quiescence through Rb/E2f is essential for neuronal survival.

Building upon the concept of non-cell cycle gene regulation, we next examined the effects of Rb-deletion in post-mitotic cortical neurons. Previous evidence had suggested that under steady-state conditions, Rb is dispensable in cortical neurons (Liu et al., 2005; Slack et al., 1998). Surprisingly, we report that acute removal of Rb results in a latent cell death response. This cell death occurs in an E2f-dependent manner, without the initial transcriptional induction of classical E2f-apoptotic regulators (Apaf1, Puma). Microarray analysis revealed that specific up-regulation of cell cycle genes occurs in Rb-deficient neurons. Examination of cell cycle re-entry revealed ectopic expression of cell cycle factors (Ki67, CycA2) and a downstream DNA damage response. Acute removal of Rb *in vivo* resulted in massive neurodegeneration with similar cell cycle re-entry and DNA damage. *This study clarifies the specific role for Rb in quiescent cortical neurons by utilizing acute paradigms. In contrast to neural precursors, we highlight the traditional role for Rb in cortical neurons in repression of cell cycle related genes and the importance this has on neuronal survival.*

5.3 Research Objective 3

Our finding that Rb plays a specific role in neuronal survival by maintaining the repressive state of cell cycle genes provides an interesting model to test mechanisms regulating gene repression. There is accumulating evidence that Rb interacts and regulates the function of chromatin modifying enzymes although, few of these interactions have been tested *in vivo* (reviewed in Dick, 2007; Talluri and Dick, 2012). Recent evidence suggests that the LXCXE-binding region, the predominant Rb interaction domain, plays a very context specific role. We therefore asked whether Rb and LXCXE directly regulate chromatin dynamics at E2f-responsive promoters in quiescent cortical neurons.

Hypothesis: Rb loss activates chromatin at E2f-responsive promoters. Chromatin activation occurs in an LXCXE-independent, E2f-dependent manner.

Our model of cortical neuron cell cycle re-entry upon Rb removal presents an intriguing model to study long-term repression by Rb. Though much has been reported about the role of pRb in chromatin remodeling, little *in vivo* physiological evidence has been put forth. We report that in the absence of Rb, E2f-responsive genes display a more active chromatin state, as evidenced by chromatin immunoprecipitation. To test the role of the LXCXE-binding domain in this regulation, we establish an acute paradigm in which we employ an $Rb^{Flox/\Delta LXCXE}$, and addition of Cre by viral or transgenic means, to assess the ability of LXCXE mutation to compensate for Rb loss. We report that Rb is able to maintain cell cycle gene repression and long-term survival in the absence of a functional LXCXE-binding domain. Using a dominant negative DP1 construct, which inhibits E2f activity and rescues viability, we show that in an Rb-deficient context, E2f activation is sufficient to prevent remodeling of chromatin to an active state. *These findings highlight the role of simple Rb-E2f interactions in the repression of cell cycle genes in quiescent cortical neurons. In addition, it presents the interesting notion that E2f-activation may have a greater role in the chromatin-state than previously thought.*

CHAPTER 2

Andrusiak MG, McClellan KA, Dugal-Tessier D, Julian LM, Rodrigues SP, Kennedy TE, Park DS, Slack RS. Direct regulation of neogenin by the pRb/E2F family plays a role in neuronal migration in the ventral telencephalon. *Mol Cell Biol*. 2011 Jan;31(2):238-47. Epub 2010 Nov 8.

The experiments were performed by MGA, with the exception of the *in situ* hybridization and explants experiments, which were performed by KAM. Experiments were conceptualized by MGA with the assistance of KAM. DDT and LMJ provided technical assistance. SR provided technical training. TEK contributed to project conceptualization, technical guidance and provided reagents. All authors contributed to critical review of the manuscript. RSS as the principal investigator provided conceptual, technical, and editorial guidance.

Rb/E2F regulates expression of neogenin during neuronal migration

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Abstract

The Rb/E2F pathway has long been appreciated for its role regulating cell cycle progression. Emerging evidence indicates that it also influences physiological events outside of cell cycle regulation. We have previously described a requirement for Rb/E2F mediating neuronal migration; however the molecular mechanisms remain unknown, making this an ideal system to identify Rb/E2F mediated atypical gene regulation *in vivo*. Here we report that Rb regulates the expression of *neogenin*, a gene encoding a receptor involved in cell migration and axon guidance. Rb is capable of repressing E2F mediated *neogenin* expression while E2F3 occupies a region containing E2F consensus sites on the *neogenin* promoter in native chromatin. Absence of Rb results in aberrant neuronal migration and adhesion in response to netrin-1, a known ligand for neogenin. Increased expression of neogenin through *ex vivo* electroporation results in impaired neuronal migration similar to that detected in forebrain specific Rb deficiency. These findings show direct regulation of *neogenin* by the Rb/E2F pathway and demonstrate that regulation of *neogenin* expression is required for neural precursor migration. These studies identify a novel mechanism through which Rb regulates transcription of a gene outside of classical E2F targets to regulate events distinct from cell cycle progression.

Introduction

The Rb pathway is best characterized for its role in regulating cell cycle progression through E2F mediated transcriptional regulation of classical cell cycle machinery target genes. Recently, however, accumulating *in vivo* and *in vitro* evidence is emerging to suggest that Rb and E2F are capable of regulating expression of atypical target genes outside cell cycle regulation in cell type specific manners (reviewed in McClellan and Slack, 2007). *In vivo*, several studies have emerged that implicate Rb and E2F interaction in novel processes beyond well characterized roles in cell cycle regulation (reviewed in Burkhart and Sage, 2008; Chen et al., 2009). In the nervous system in particular, we have recently shown that a Rb E2F3 interaction mediates migration of a subpopulation of GABAergic interneurons (McClellan et al., 2007). In the same study, we also observed deregulation of a number of genes with known roles in neuronal migration in cell populations lacking Rb, suggesting a role for E2F3 in regulating transcription of novel targets (McClellan et al., 2007). A second cell cycle independent role for E2F3a in regulating Rb mediated interneuron differentiation was also reported in the retina (Chen et al., 2007). Thus far, *in vivo* studies have failed to identify the mechanism through which these cell cycle independent processes occur.

In parallel, *in vitro*, several microarray studies examining changes in gene expression in response to various models of deregulated E2F expression have each identified groups of overlapping novel target genes with well characterized roles in differentiation, development, and migration (Black et al., 2005; Dimova et al., 2003; Ishida et al., 2001; Ma et al., 2002; Muller et al., 2001; Polager et al., 2002; Young et al., 2003). More recently, ChIP-on-chip studies have identified putative E2F binding sites within the promoters of a number of genes unrelated to the cell cycle (Balciunaite et al., 2005; Bieda et al., 2006; Cam et al., 2004; Jin

et al., 2006; Ren et al., 2002; Weinmann et al., 2001; Weinmann et al., 2002). Finally, using an approach whereby novel genes induced by E2F1 are identified based on subtraction screening, genes with known roles in differentiation and migration were identified as being directly induced by E2F1, in a cell cycle independent manner (Iwanaga et al., 2006). Thus, these data provide evidence that our understanding of the significance of Rb/E2F function should be expanded to include transcriptional regulation of genes beyond the well characterized subset of targets that regulate the cell cycle.

Our identification of a role for Rb/E2F3 in mediating neuronal migration represents an attractive model to identify novel cell cycle independent E2F target genes in the context of an *in vivo* physiological function (Ferguson et al., 2005; McClellan et al., 2007). Given our previous observations revealing (i) deregulation of a number of genes in families of known chemotactic ligands and receptors implicated in neuronal migration in the absence of Rb; and, (ii) the cell autonomous requirement for Rb in neuronal migration; we hypothesized that Rb/E2F may modulate the transcription of novel target genes involved in neuronal migration. We focused our efforts on *neogenin*, a receptor for the netrin and RGM families of chemotropic ligands (reviewed in De Vries and Cooper, 2008). Notably, neogenin is highly expressed by a subpopulation of interneurons migrating from the ventral forebrain and has been independently identified, in an *in vitro* overexpression system, as an E2F regulated gene (Iwanaga et al., 2006; McClellan et al., 2007). Here we report that Rb directly regulates the expression of a non-traditional target, *neogenin*. Rb is capable of repressing E2F mediated transcription of *neogenin* while E2F3 binds to a region containing a conserved E2F consensus site on the *neogenin* promoter in native chromatin. The absence of Rb results in aberrant neuronal migration and adhesion in response to the neogenin

ligand, netrin-1. Finally increased expression of neogenin through *ex vivo* electroporation results in impaired neural precursor migration similar to that observed in forebrain specific Rb deficiency. From these findings, we show direct regulation of *neogenin* by the Rb/E2F pathway and demonstrate that correct regulation of *neogenin* expression is required for neural precursor cell migration. Through these studies we identify a novel mechanism through which Rb interacts with E2F to regulate transcription of genes outside of classical E2F targets to influence biologically relevant events distinct from cell cycle progression.

Materials & Methods

Mice

Telencephalon specific Rb deficient mice were generated by crossing floxed Rb-F19 (Marino et al., 2000; Vooijs et al., 1998) and Foxg1-cre mice (Hebert and McConnell, 2000), and were genotyped according to standard protocols with previously published primers (Ferguson et al., 2005; Ferguson et al., 2002). For embryonic time points, the time of plug identification was counted as embryonic day (E) 0.5. For all experiments littermate Rb conditional mutants ($Rb^{flox/flox}$ Foxg1-$cre^{+/-}$) and double heterozygous controls ($Rb^{flox/+}$ Foxg1-$cre^{+/-}$) were compared. Due to Rb autoregulation (Shan et al., 1994), Rb expression in heterozygous mice is similar to wild type controls. All experiments were approved by the University of Ottawa's Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

Western Blot

Protein was isolated from neurospheres by treating cells with lysis buffer (10 mM Tris, 0.15 M NaCl, 1 mM EDTA, 0.4 mM Sodium Vanadate and 0.5% Triton-X). Cells were incubated on ice for 20 min, followed by a 10 min centrifugation at maximum speed to remove debris. Western blots were performed as previously described (Ferguson et al. 2000) with antibodies directed towards neogenin (H-175, Santa Cruz), Rb (Pharmingen), p130Cas (BD Biosciences) and beta-actin (Sigma). Immunoblots were performed on three independent samples and quantified using ImageJ software.

Tissue Preparation and In situ hybridization

Tissue was dissected, fixed, cryoprotected, and sectioned as previously described (McClellan et al., 2007). Non-radioactive *in situ* hybridization and digoxygenin probe labeling was performed according to previously described protocols (Wallace and Raff, 1999). Neogenin, DCC, netrin, and RGMa, were generous gifts of Dr. Helen Cooper- University of Queensland (Gad et al., 1997), Dr. Elke Stein- Yale University, and Dr. Silvia Arber, University of Basel (Niederkofler et al., 2004). All results shown are representative of those obtained with a minimum of 3 independent animals, n=3.

Transcription Factor Binding Sites

The 5' region of the mouse Neo1 locus containing the intergenic region, the untranslated region, and exon 1 was analyzed for putative E2F binding sites using the TRANSFAC Professional Library V10.2 through Mulan/MultiTF (http://rvista.dcode.org). All sites identified in Mulan were manually examined for their similarity to the consensus (TTTSSCGC) and nonconsensus (BKTSSCGS) E2F motifs.

Chromatin Immunoprecipitation

Neurosphere cultures were prepared from CD1 embryos (Charles River) at embryonic day 14.5. Proliferating neurospheres were triturated, cross-linked with formaldehyde, lysed, sonicated, and centrifuged at 14,000 x *g* to remove cellular debris. Each immunoprecipitation was performed using 2 μg of antibody. Antibodies against E2F3 (sc-878), E2F1 (sc-193) and normal rabbit immunoglobulin G (sc-2027), were obtained from Santa Cruz Biotechnology. Immunocomplexes were captured using protein A/G9 Sepharose beads and washed extensively, and cross-links were reversed overnight, followed by treatment with RNase A at 37°C for 1 h and proteinase K at 65°C for 30 min. The purified

DNA was examined by PCR using primers designed around the E2F consensus sites at -44bp and -821bp in the 5' region of the neogenin gene. Immunoprecipitations were performed from three cultures obtained from three independent animals.

DNA constructs

The *neogenin* construct was PCR amplified from pSecTagA-Neogenin (rat) using: Forward: GAACTGCAGACCATGGAAGAAAGA, Reverse: CTTCTGCAGGTTGCCCTCTAGCTAG. PCR fragment was then subcloned into pCIG2. The 5' regulatory region of *neogenin* was PCR amplified from embryonic genomic DNA. Primers were designed as follows with flanking Xho1 restriction sites inserted: Full length Forward AGACTCGAGGAGGTGCAGAGGAGTCGC, Full length reverse TGTCTCGAGGTTGAAAAACCAATTCCCG. To create 5' truncations the following primers were used with full length reverse primer: FTrunc247 AGACTCGAGAGCCGGGGGGTGG, FTrunc618 AGACTCGAGAAGCGATCCGCCTCCT. To create the 3' truncation the following primer was used with full length forward primer: RTrunc 247np TGTCTCGAGCCACCCCCCGGCT. The 247-618bp construct was made using the FTrunc247 and RTrunc 618bp TGTCTCGAGGCGGATCGCTTCTCC. PCR products was digested with Xho1 and ligated into pGL4.24 (Promega). Sequence was confirmed by DNA sequencing (StemCore, University of Ottawa).

Luciferase Reporter Assays

HEK293T cells were transfected using Lipofectamine (Invitrogen) as per manufacturers' protocol. Briefly cells were transfected with 500 ng pGL4.24, pGL4.24-Neogenin, or

pGL4.24-NeoTruncations, 10 ng E2F3, 300 ng Rb and total transfected plasmid normalized with pcDNA3.1. Transfection efficiency was normalized using 10 ng of pRL renilla expressing vector. Cells were lysed 24h post-transfection and examined by spectrophotometer (LMaxII, Molecular Devices) for luciferase expression by Dual-Glo Luciferase (Promega).

In vitro explant cultures

In vitro explant cultures were performed as described (Colombo et al., 2007; Kennedy et al., 1994; Metin et al., 1997; Pozas and Ibanez, 2005; Pozas et al., 2001) with some modifications. Briefly, brains were removed from E14.5 embryos in L15 (Gibco) media and medial ganglionic eminence (MGE) dissected as described (Ferguson et al., 2005) followed by dividing explants into pieces approximately 200 µm in diameter with sharpened tungsten needles. MGE explants were then transferred into collagen (Inamed BioMaterials PureCol™Cat No. 5409) inside culture dishes and allowed to solidify for 40 min prior to addition of Neurobasal media supplemented with FBS. Purified netrin-1 was added to the explants culture media at a final concentration of 200 ng/ml.

Explants cultured alone were grown *in vitro* for 24 hr. Images were captured with a Zeiss Axioscope microscope. For quantification of cell migration in collagen, total number of cell bodies migrating from explants was counted. 2-4 explants per embryo were measured and values averaged. Two-tailed t-tests were performed to compare mean migration between genotype, or treatment groups. Differences were considered significant at $p < 0.05$.

Neural Progenitor Cultures

Pregnant mice were euthanized at gestation day 14.5, embryos removed and the ganglionic eminences isolated by microdissection. For determination of cell proliferation and cell death in single cell preparations, ganglionic eminences were dissociated and equal cell numbers plated on poly-D-Lysine and laminin-1 coated dishes in duplicate. Cells were cultured in Neurobasal media supplemented with 0.5 mM L-glutamine, 1% N2, 2% B27, 10 ng/ml FGF2 and 20 ng/ml EGF in either the presence or absence of netrin-1 at 200 ng/mL. Cells were treated with BrdU at a final concentration of 10 μg/ml for 45 min prior to fixation. After 24 hrs, cells were fixed for 15 min in 4% PFA and then treated sequentially with 2 N HCl for and 0.01 M NaB_4O_7 followed by BrdU immunohistochemistry (anti-mouse BrdU, 1:100, BD Biosciences, San Jose, CA) and Hoechst nuclear staining. Total cells and BrdU-positive cells were counted in 3 microscope fields per duplicate well. Rates of proliferation were obtained by calculating the proportion of BrdU-positive cells relative to the total cell number. Fold increase in proliferation in response to netrin-1 was calculated for each genotype by dividing the percent proliferating cells in the presence of netrin-1 by the percentage of proliferating cells in the absence of netrin-1 (Three separate embryos were analyzed in quadruplicate for both control and conditional mutant embryos). For Hoechst labeling, dead cells were identified by the characteristic condensation of chromatin. Fold increase in apoptotic nuclei was calculated in an analogous manner to cell proliferation (Three separate embryos were analyzed in quadruplicate for both control and conditional mutant embryos).

Substrate-bound Adhesion Assay

To assess cell adhesion in the presence of netrin-1 a substrate bound adhesion assay was performed as described (Shekarabi et al., 2005). Briefly, 20 µl of 0.1% nitrocellulose (Hybond ECL; Amersham Biosciences) dissolved in methanol was dried on the bottom of a four-well plate. Plates were then incubated with either HBSS or 2 µg/ml netrin-1 in HBSS for 2 hr at room temperature. Wells were then blocked for 1 hr with 1% BSA (Fisher Scientific) in HBSS and then again with 1% heparin (Sigma) in HBSS. 2.5 x 10^5 cells from dissociated ganglionic eminences were plated in Neurobasal (Gibco) supplemented with 2% B-27 and 2 mM glutamine. Cells were cultured for 2 hr at 37°C, 5% CO_2, gently washed once with PBS, and fixed with 4% PFA in PBS overnight. Nuclei were labelled with 0.5 µg/ml Hoechst 33258 (Sigma) in PBS for 30 min. Experiments were performed on 4 wild-type and 3 mutant embryos. Paired, two-tailed t-tests were performed to compare genotypes, with differences considered significant at $p < 0.05$.

Ex vivo cortical electroporation

Cortical electroporation and *ex vivo* slice culture was performed as described (Ferguson et al., 2005; Hand et al., 2005; Polleux and Ghosh, 2002), with some modifications. Briefly, pregnant female mice (Charles River) were euthanized at E15 with a lethal injection of sodium pentobarbital. Embryos were removed, decapitated and a 2 µg/ µL solution of pCIG-Neogenin or empty pCIG vector (supplemented with 0.5% Brilliant Blue, FCF for visualization) was injected, using a Picospritzer II (General Valve Corporation) into the lateral ventricles. Brains were subjected to 10 pulses at 70V using an ElectroSquarePorator ECM830 (Btx, Genetronics San Diego, CA). Brains were then isolated and embedded in

low melting point agarose. Agarose-embedded brains were sectioned coronally into 250 μm sections on a Leica VT1000S vibratome. Brain sections were collected and plated on poly-*L*-lysine/laminin-coated filter-membrane inserts placed on top of culture media in each well of a 6-well dish as described (Ferguson et al., 2005; Polleux and Ghosh, 2002). Slices were then cultured for 72 hrs to assess the degree of migration. Two sections from 5-6 embryos from 3 litters were measured and values averaged. Migration was assessed by measuring the total area occupied by GFP positive cells at both 24 hr and 72 hr time points. Degree of migration in each condition was assessed by subtracting the area occupied at 24 hrs from the area occupied at 72hrs and then dividing this value by the initial area to obtain a percentage of migration over time. Degree of migration in neogenin expressing samples relative to control was obtained by dividing percent migration in neogenin over percent migration over control. Two-tailed t-tests were performed to compare migration between control and neogenin overexpression, with differences considered significant at $p < 0.05$.

Results

Rb deficiency results in specific deregulation of *neogenin* expression *in vivo*

Our previous studies have described a physiological requirement for Rb interacting through E2F to mediate nervous system development (McClellan et al., 2007). Furthermore, we reported that Rb deficiency results in increased expression of mRNA encoding neogenin, a receptor involved in regulating axon guidance and cell migration during neural development (reviewed in De Vries and Cooper, 2008; Yamashita et al., 2007). The physiological significance of Rb mediated regulation of *neogenin* expression on nervous system development, however, is unknown. To assess the contribution of neogenin to Rb mediated nervous system development we first asked if deregulation is unique to *neogenin* or extends across the family of neogenin ligands and related receptors. To address this question in the telencephalon, sections from Foxg1-cre conditional Rb mutants were subjected to *in situ* hybridization to examine the expression profiles of neogenin, the closely related receptor Deleted in Colorectal Cancer (DCC), and the known neogenin ligands Repulsive Guidance Molecule (RGMa) and netrin-1 (Figure 2.1A). Consistent with our previous microarray and *in situ* hybridization findings (McClellan et al., 2007), we detected increased neogenin expression throughout the ventral and dorsal telencephalon in conditional Rb mutants. In the ganglionic eminence, a source of migrating interneurons, no difference was detected in the expression pattern of DCC, netrin-1 and RGMa between control and conditional Rb mutants (n=3) (Figure 2.1A). These expression patterns parallel other studies that have shown neogenin and netrin-1 overlapping protein expression in the ganglionic eminence (Fitzgerald et al., 2006; Stanco et al., 2009). Thus, of the members of the netrin signalling pathway, only a significant change in the expression of neogenin was

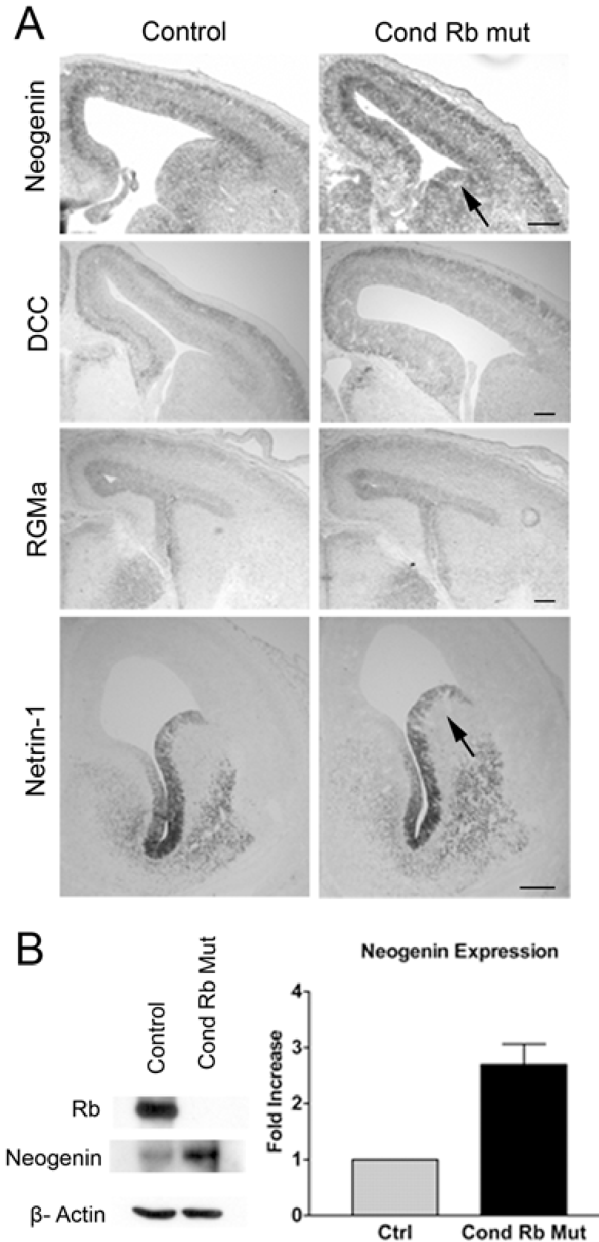

Figure 2.1: **Neogenin is upregulated in the absence of Rb in the developing forebrain**. A) In the absence of Rb (*Foxg1-Cre/+;RbloxP/loxP)*, increased neogenin expression is detected in the subventricular zone, cortex and striatum when compared with control (*Foxg1-Cre/+;RbloxP/+)*. In situs were performed on three or more independent samples for DCC, RGMa, netrin1, neogenin. Arrows indicate regions of overlapping expression between neogenin and netrin-1 in the ganglionic eminence. B) Western blot analysis was performed on neural precursor cells isolated from E14.5 Rb conditional mutants and control embryos. Note efficient recombination of the floxed Rb allele, showing no detectable Rb. Rb mutants showed an upregulation of neogenin compared to levels in the control. Densitometrric quantification of neogenin expression detected by western blot was performed using ImageJ from three independent experiments. Significance was determined through a paired two-tailed T test for control and conditional Rb mutant *$p < 0.05$.

detected within the ventrally derived population of neural precursor cells. To validate increased neogenin levels identified by *in situ* hybridization, we assessed expression at the protein level within the migrating cell population. Total protein was extracted from the population of ventrally derived neural progenitor cells from 3 separate embryos for each genotype and a similar increase in neogenin protein was identified in conditional Rb mutants (Figure 2.1B). Efficient excision of the Rb allele in the context of primary ventral neural precursors was confirmed by protein levels (Figure 2.1B). Together these data support the hypothesis that Rb may play an important role in regulating expression of neogenin, a non traditional E2F target gene.

E2F3 binds the 5' locus of neogenin in vivo

Recent studies have reported that *neogenin* is among a novel class of atypical E2F target genes regulated in a cell cycle independent manner (Iwanaga et al., 2006). E2F1 was shown to be capable of directly inducing neogenin expression outside of growth stimulation, however, no classical E2F binding (TTTSSCGC) site was observed within the 5' region (Iwanaga et al., 2006). With the implementation of new bioinformatic techniques, a new broad E2F consensus binding site(s) (BKTSSCGS) has recently been characterized (Rabinovich et al., 2008). We asked whether E2F could be mediating neogenin expression through one of these newly defined E2F sites contained in its 5' regulatory region. Using Mulan/rVista software, the 5' region of the mouse *neogenin* gene was examined for the presence of broad spectrum BKTSSCGS E2F motifs (Rabinovich et al., 2008). Consistent with previous reports, no classical E2F binding sites could be found in the mouse *neogenin* promoter (Iwanaga et al., 2006). Closer examination revealed ten broad E2F binding sites

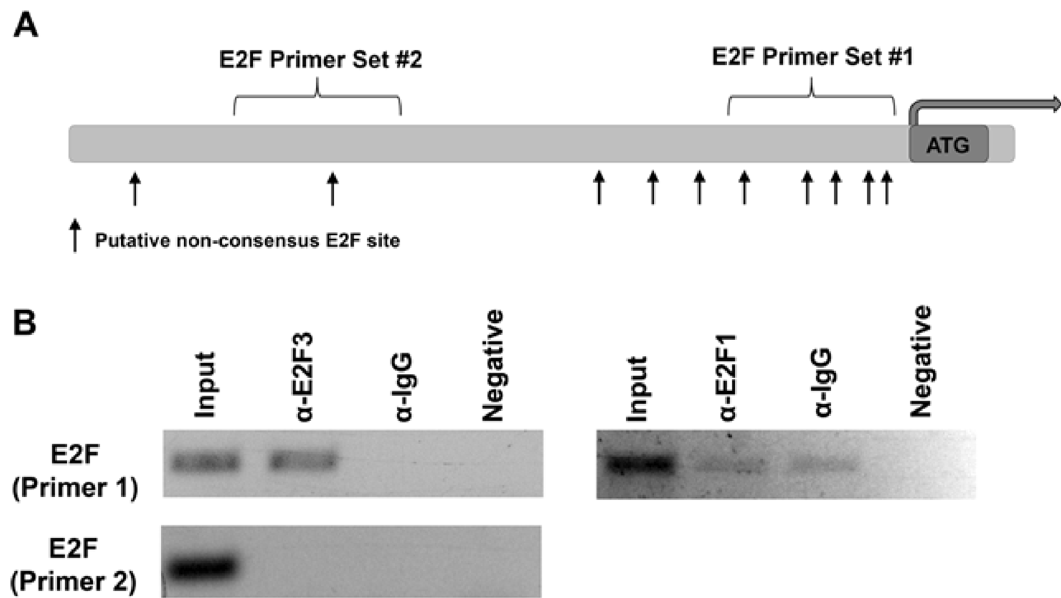

Figure 2.2**: E2F3 interacts with a region containing multiple putative E2F sites within the 5' regulatory region of neogenin.** A) Putative E2F sites were identified in the 5' region of the mouse (mm9) Neo1 gene using Mulan/rVista software and confirmed by manual sequence analysis (BKTSSCGS). B) ChIP was performed on neurospheres isolated from the ganglionic eminence of E14.5 wild-type embryos. Immunoprecipitation was performed using an antibodies specific to E2F3 or E2F1 followed by PCR amplification of the indicated regions in the 5' regulatory region of the neogenin gene. ChIP was performed on three independent cultures per condition. An interaction was only detected with E2F3 at the region containing multiple clustered sites, the putative site at 852 bp showed no specific binding. Immunoprecipitations were performed on independent cultures from three animals.

located within 1.23kb upstream of the translational start site. The majority of these putative E2F sites are clustered within the first 600bp upstream of the ATG (Figure 2.2A).

Prior studies examined the regulation of neogenin expression by E2F1, however as we have previously described unique roles for E2F3 in nervous system development (McClellan et al., 2007; McClellan et al., 2009) we asked whether E2F3 interacted with the 5' regulatory region of the *neogenin* gene in the context of native chromatin. To address this question, we performed chromatin immunoprecipitation (ChIP) in primary neural progenitor cultures to see if E2F3 associated with the regions containing atypical E2F sites. Chromatin was immunoprecipitated with antibodies to E2F3 followed by PCR with primers designed around the cluster of E2F sites immediately upstream of the translation start and the region containing only a single site 852bp upstream (Figure 2.2A-B). ChIP with the primer set surrounding the cluster demonstrated enrichment of E2F3 binding; with no detectable E2F3 binding at the site at -852bp (Figure 2.2B). Consistent results were obtained from 3 independent primary cultures. Previous studies using a subtractive microarray analysis revealed that E2F1 could induce neogenin expression in rat embryonic fibroblasts; however, a direct interaction in the *neogenin* regulatory regions was not shown (Iwanaga et al., 2006). We therefore asked if E2F1 might physically interact with the putative promoter region of *neogenin*. To this end we performed ChIP with an antibody directed to E2F1 and examined the region in which E2F3 binding was detected. No enrichment was found in this region (Figure 2.2B), suggesting that E2F1 does not interact with the *neogenin* promoter in precursors from the ventral forebrain. These results demonstrate that, in the context of neural precursor cells, E2F3 specifically binds the *neogenin* promoter at the region (-600 to

ATG) encompassing the multiple putative E2F consensus sites in an *in vivo* context and is consistent with the hypothesis that E2F3 is capable of modulating neogenin gene expression.

Rb/ E2F regulates transcriptional activity at the 5' neogenin regulatory region

Given that we observe deregulated neogenin expression in the absence of Rb, we next asked whether Rb regulates this expression through its interaction with E2F. To address this question we performed *in vitro* luciferase reporter assays in HEK293T cells. Ideally, these studies should be performed in primary systems, however, over expression of E2Fs 1 or 3 induces a rapid and robust apoptotic response in embryonic tissue and in primary neural precursor cells. As HEK293T cell lines express E1B preventing apoptosis (White et al., 1992), they withstand overexpression of 'activating' E2F constructs without undergoing cell death. Thus, all reporter assays were performed in HEK293T cell lines. The *neogenin* promoter region was amplified from embryonic genomic DNA with primers designed to flank a 1.23 kb region containing the putative E2F binding sites (Figure 2.3A). The fragment was then subcloned into a luciferase reporter vector (pGL4.24) which was subsequently transfected into HEK 293 cells.

In the absence of exogenous E2F, the *neogenin* promoter displayed a minimal level of activation (Figure 2.3B). Upon addition of E2F3, however, we observed a strong 13.3 fold increase in luciferase activity demonstrating that E2F3 is capable of transcriptionally activating the *neogenin* promoter. We next asked if Rb is capable of repressing E2F mediated activation of the *neogenin* promoter. Upon co-transfection of E2F3 and Rb expression plasmids, E2F3 mediated activation of the *neogenin* promoter was repressed

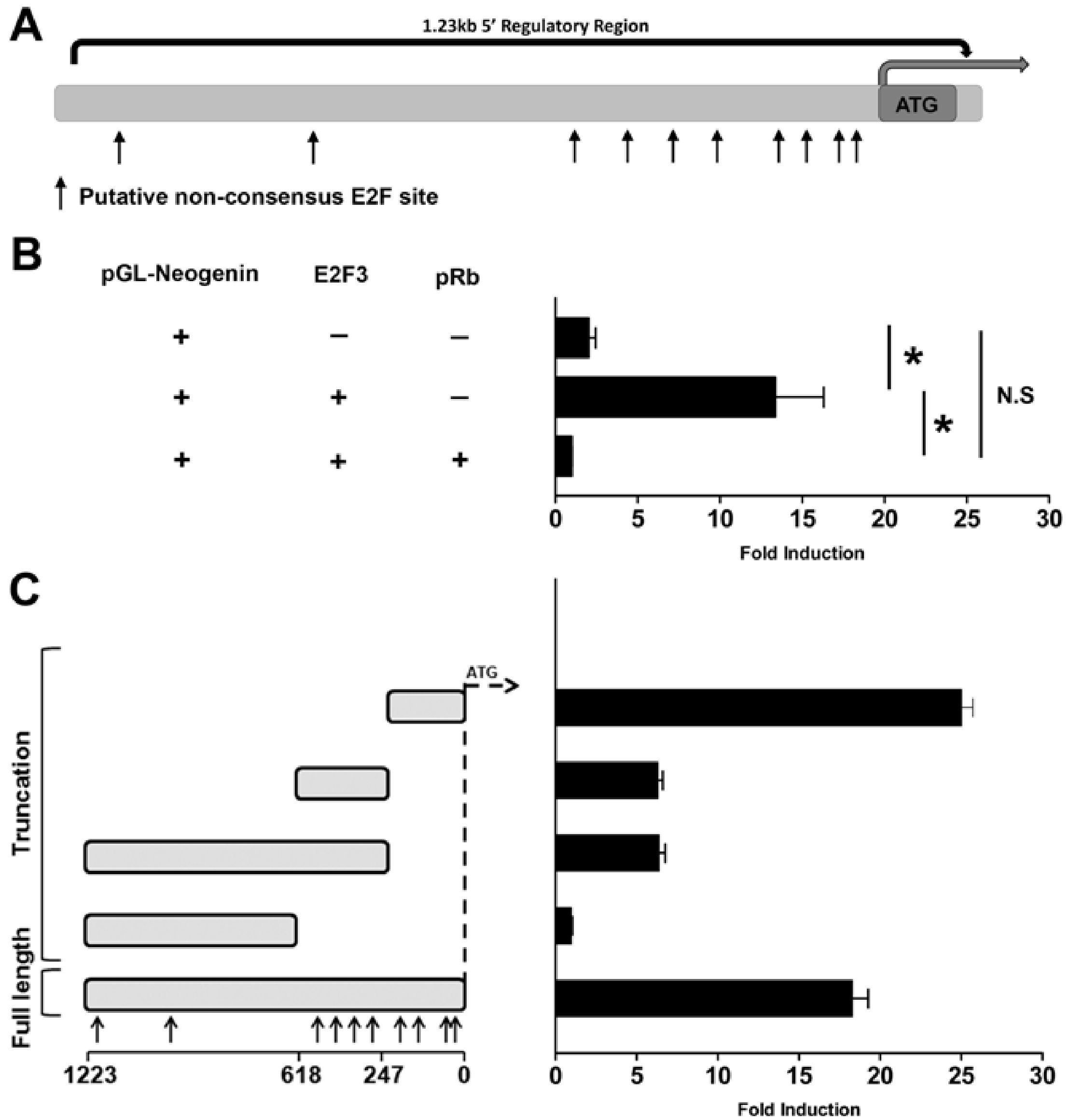

Figure 2.3: **The 5' neogenin promoter is responsive to Rb/E2F regulation.** A) Schematic of the 5' region of the neogenin gene. The 1.23kb region isolated and cloned into the pGL4.24 vector contains ten putative E2F binding sites identified using Dcode/Mulan software. B) Dual-Glo Luciferase (Promega) promoter assays in HEK293T cells utilizing the neogenin promoter reveal that the addition of E2F3 induces a thirteen-fold induction of neogenin promoter activity. When stimulated with Rb and E2F3 the activation is eliminated and luciferase levels return to that of promoter alone. C) Luciferase analysis of truncations of the 5' region of the neogenin gene. Activity is ablated upon removal of the initial 618bp. The region between 247-618bp results in slight E2F3 responsiveness, however, the first 247bp recapitulates full-length promoter activity. * $p < 0.05$, N.S corresponds to non-significant difference.

back to basal levels (Figure 2.3B). Together these results demonstrate that Rb acts to repress E2F3 mediated activation of the *neogenin* promoter through interaction in the 5' regulatory region.

We next sought to determine the specific region in the neogenin promoter in which E2F3 was binding to activate transcription. Examination of the 5' proximal promoter for both classical/nonclassical E2F binding motifs revealed 10 putative sites within the 1.23kb examined. To define the essential regions required for E2F-mediated activation, we created multiple truncation constructs at roughly 250bp intervals from both the 5' and 3' ends of the 1.23kb promoter construct (Figure 2.3C). While constructs lacking the regions upstream of -247bp, had no effect on E2F responsiveness, absence of the region from the ATG to -618bp, abolished the ability of E2F to activate transcription (Figure 2.3C). This region was most enriched with 8 putative E2F sites and suggests that E2F3 is binding one or more of the several clustered sites in this region. To more precisely identify the sites, 2 further truncation constructs (0-247bp, 247-618bp) were made lacking each of the two clusters of E2F sites within the first 618bp of the promoter (Figure 2.3C). Introduction of the construct containing only the region encompassing -247-618bp, upstream of the promoter (Figure 2.3C), resulted in slight activation in response to E2F (Figure 2.3C). This result recapitulated the slight E2F responsiveness observed in the construct lacking the first 247bp upstream of the ATG. To determine if the first 247bp could confer E2F-mediated activation, this 247bp fragment alone was ligated into the reporter construct and full E2F responsiveness, equivalent to the full-length construct, was obtained. These results demonstrate that the cluster of E2F sites contained in the 247bp upstream of the ATG are essential for neogenin promoter activation, consistent with the region identified by our E2F3

ChIP (Figure 2.2B). These findings support the conclusion that Rb, acting through E2F3, directs the expression of neogenin, an atypical E2F target gene, that functions outside of cell cycle progression.

Rb deficiency results in aberrant neuronal migration in the presence of netrin-1

We next sought to determine the functional consequences of deregulation of *neogenin* expression as a result of the ablation of the Rb gene. We hypothesized that if deregulated expression of *neogenin* contributes to the aberrant migration of ventrally derived neurons in the conditional Rb mutant, then neural precursor cells should elicit an aberrant response in the presence of neogenin ligand. During mammary gland development, netrin-1-neogenin interactions have been shown to be crucial for proper stabilization of the multipotent progenitor cell layer (Fitzgerald et al., 2006; Srinivasan et al., 2003). This interaction may play an analogous role during tangential migration in the developing forebrain. We therefore determined if netrin-1 is capable of influencing migration of MGE derived cells under wild-type conditions. To effectively address this question, we employed a reductionist *in vitro* approach using primary neural precursor explants cultured in a collagen matrix. This approach created a defined extracellular environment containing Netrin1 alone, and allowed us to determine the effect of the neogenin ligand, netrin in the absence of other competing signals known to influence migration (Marin and Rubenstein, 2001). Explants of ventral ganglionic eminence were microdissected from control and mutant E14.5 cerebral hemispheres and then cultured for 24 hrs supplemented with netrin-1, after which cell migration from the explant was quantified. We assessed the relative contribution of netrin-1, a ligand demonstrated to elicit neogenin dependent

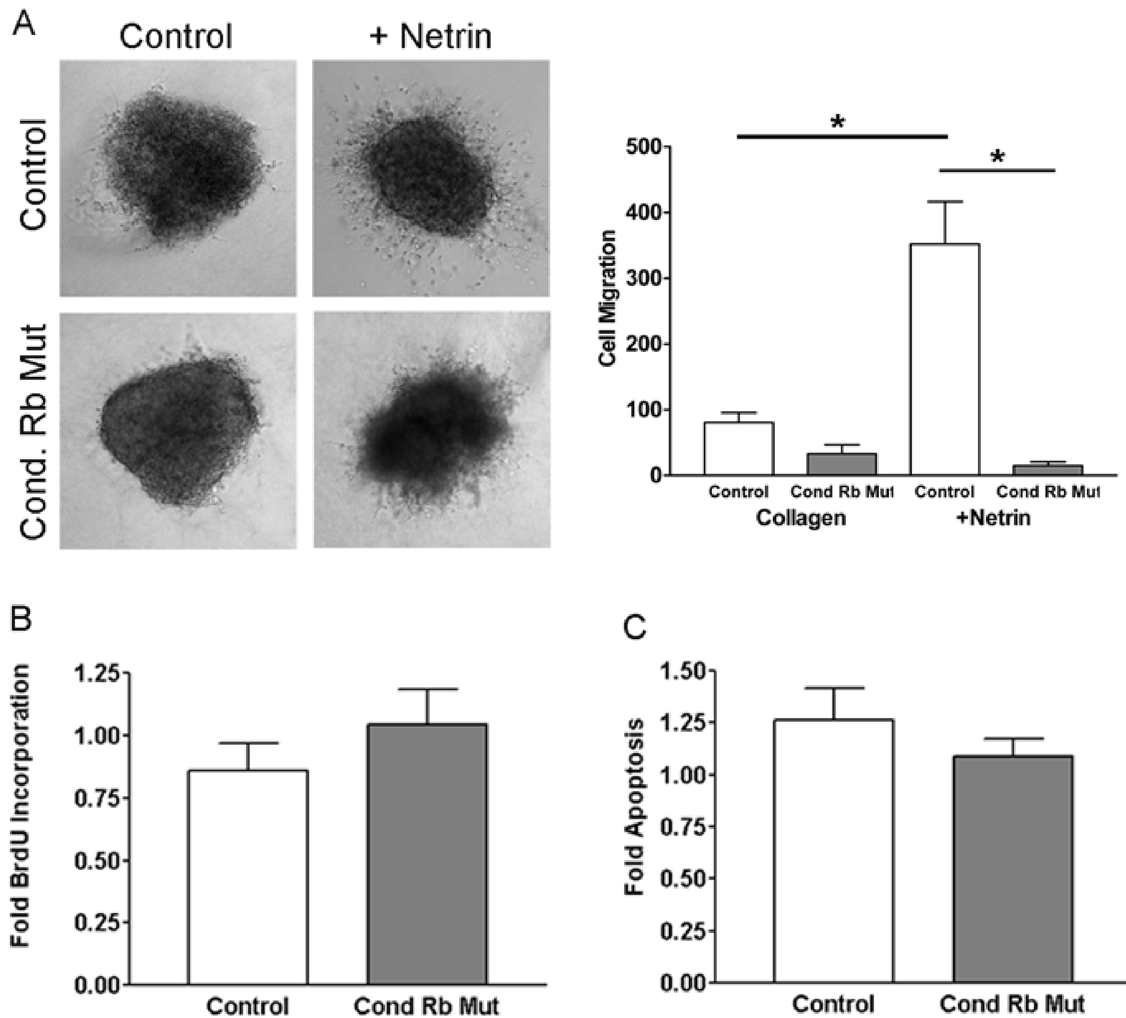

Figure 2.4: **Conditional Rb mutants display a defective migratory response to netrin-1.** (A) Control (*Foxg1-Cre/+;RbloxP/+)* and conditional Rb mutants (*Foxg1-Cre/+;RbloxP/loxP)* MGE explants were cultured in collagen in the absence or presence of recombinant netrin-1. Migration was quantified by counting the individual cell bodies migrating from each explant. Bars represent mean of the average number of cells migrating from an individual explant ± SEM. While control cells exhibit a nearly fourfold increase in migration in the presence of netrin, no difference is observed in conditional Rb mutants between presence and absence of netrin. Significance was determined through a paired two-tailed T test for explants of the same genotype and a two-tailed t-test for explants of different genotype. * $p < 0.05$ (n= 4 embryos per treatment, per genotype, 2-3 explants examined per embryo). (B,C) Cells from the ganglionic eminence of control and conditional Rb mutants were dissected and cultured as single cell preparations in the presence or absence of netrin-1. Quantification of the proportion of cells in S-phase (BrdU) or dying (Hoescht) reveals no change upon addition of netrin-1 in either genotype. Three separate embryos were analyzed in quadruplicate for both control and conditional Rb mutant.

chemoattractrant responses in the developing nervous system. Explants were cultured in collagen, a matrix suitable for assessing chemoattractant responses (Kennedy et al., 1994; Metin et al., 1997). Both control and conditional Rb mutant explants cultured in collagen alone exhibited modest numbers of cells migrating, with no appreciable difference in migration from either type of explant. In the presence of netrin-1, however, a clear difference was observed (Figure 2.4A). While control explants exhibited a four-fold increase in migration in the presence of netrin-1, there was no significant difference in the number of cells migrating from conditional Rb mutant explants cultured in the presence or absence of netrin-1 (Figure 2.4A).

Netrin-1 has been hypothesized to mediate cell proliferation and cell death (reviewed in Cirulli and Yebra, 2007; Mehlen and Furne, 2005). We therefore verified that the differences detected in migration were not due to either of these processes. To dissect the potential contribution of altered proliferation or cell death, netrin-1 treatment was performed under conditions that recapitulate those used in our *in vitro* explant culture, in order to act as a control for proliferation and cell death under those specific conditions. To examine proliferation, cultures were treated with bromodeoxyuridine (BrdU) and the proportion of cells in S-phase of the cell cycle counted. In 3 independent control and mutant cultures, netrin-1 treatment did not significantly impact the number of proliferating cells (Figure 2.4B). Assessment of chromatin condensation revealed no significant change in cell death between control and mutant cultures upon addition of netrin-1 (n = 3) (Figure 2.4C). These results suggest that the increased number of cells migrating in response to netrin-1 is not the consequence of increased cell proliferation, nor can the absence of migration in the conditional Rb mutant be attributed to increased cell death. Thus, this data supports a model

whereby netrin-1 is capable of influencing migration of ventrally derived progenitors, an effect that is not observed in the conditional Rb mutants. These results suggest that ventrally derived progenitors from Rb mutants are inherently unable to elicit the appropriate migratory response to netrin-1 itself.

Increased adhesion to substrate-bound netrin-1 in conditional Rb mutants

A previous study demonstrated that netrin-1 and neogenin interact to mediate adhesion in the mammary gland (Srinivasan et al., 2003). Given that we observe reduced migration in response to netrin-1 in the conditional Rb mutant, where *neogenin* expression is increased, we determined if the increased amount of neogenin present would increase adhesion of neural precursor cells. To address this, adhesion assays were performed which have been previously used to assess netrin-neogenin – mediated adhesion in fibroblasts (Srinivasan et al., 2003) and adapted for neural precursor cells (Shekarabi et al., 2005). Using this assay, we examined the capacity of ventrally derived precursors from control and conditional Rb mutants to adhere specifically to immobilized netrin-1. The ventral telencephalon from E14.5 control and conditional Rb mutants was dissected and dissociated into single cell suspensions. Cells were quantified and then equal numbers were plated and allowed to adhere to culture dishes preadsorbed with nitrocellulose alone or with netrin-1 and nitrocellulose. After 2 hrs, cells were washed, fixed and cell adhesion was quantified. Data was represented as a fold increase in adhesion upon netrin-1 treatment to eliminate the experimental variability observed from each independent assay. Results from independent experiments, however, produced highly consistent results (shown Table 1). In the presence of netrin-1, conditional Rb mutants displayed a 3.6 fold increase in adhesion, whereas control littermates displayed a significantly smaller 2.5 fold increase (Figure 2.5). Our

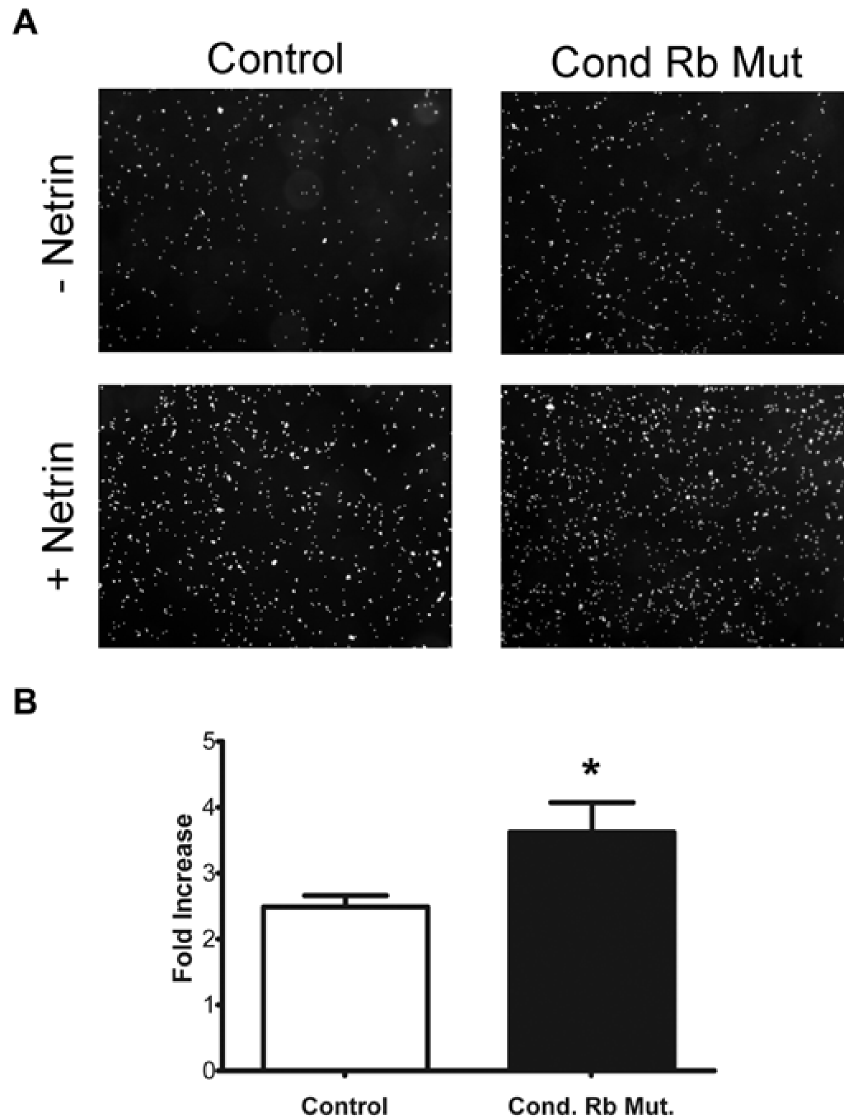

Figure 2.5: **Rb deficient neural precursors show an increased propensity to adhere to substrate bound netrin-1.** A) Cells from the ganglionic eminence of control (*Foxg1-Cre/+;RbloxP/+)* and conditional Rb mutants (*Foxg1-Cre/+;RbloxP/loxP)* were dissected at E14.5. Cells were allowed to adhere to netrin-1 or non-coated wells for 2 hrs, fixed and stained. Cells were then imaged and nuclei counted. In the absence of Rb, cells from the ganglionic eminence display more significant adherence to netrin-1 than cells from control littermates. B) Fold increase represents the increase adhesion from non-coated to netrin-1 coated wells. Error bars represent standard error of the mean (SEM). n=4 for control and n=3 for conditional Rb mutant. Significance was determined through a paired two-tailed T test for control and conditional Rb mutant cultures* $p < 0.05$.

Experiment	Embryo	Genotype	No Netrin (AvgCells/Field)	Netrin (AvgCells/Field)	Fold Increase
CTL	MGA57	*Foxg1-Cre/+;RbloxP/+*	382	925	2.421466
CTL	MGA62	*Foxg1-Cre/+;RbloxP/+*	235.5	513	2.178344
CTL	MGA132	*Foxg1-Cre/+;RbloxP/+*	121.5	285.5	2.349794
CTL	MGA133	*Foxg1-Cre/+;RbloxP/+*	127.75	291.4	2.281018
RB-/-	MGA134	*Foxg1-Cre/+;RbloxP/RbloxP*	100	316.25	3.1625
RB-/-	MGA136	*Foxg1-Cre/+;RbloxP/RbloxP*	113.5	504.75	4.447137
RB-/-	MGA59	*Foxg1-Cre/+;RbloxP/RbloxP*	464	1635	3.523707

Table 2.1: **Rb deficient neural precursors show an increased propensity to adhere to substrate bound netrin-1.** A) Cells from the ganglionic eminence of control (*Foxg1-Cre/+;RbloxP/+)* and conditional Rb mutants (*Foxg1-Cre/+;RbloxP/loxP)* were dissected at E14.5. Cells were allowed to adhere to netrin-1 or non-coated wells for 2 hrs, fixed and stained. Cells were then imaged and nuclei counted. The total number of nuclei per field were averaged for each condition. In the absence of Rb, cells from the ganglionic eminence display more significant adherence to netrin-1 than cells from control littermates. Fold increase represents the increase adhesion from non-coated to netrin-1 coated wells.

findings suggest that Rb deficient neural precursor cells have increased adhesive properties, consistent with previous findings revealing a role for netrin-neogenin in mediating cellular adhesion (Shekarabi et al., 2005; Srinivasan et al., 2003). Given the elevated levels of *neogenin* expression detected, increased adhesion in response to netrin-1 may be a key contributing factor to the migration defect present in Rb deficient brains.

Increased neogenin impedes neuronal migration

While we have demonstrated that Rb is capable of regulating neogenin transcription through E2F in the developing nervous system, the consequence of increased neogenin expression remains unknown. We therefore asked if upregulation of neogenin as found in the Rb deficient forebrain was sufficient to disrupt the migration of MGE derived neurons. To determine whether increased neogenin expression could perturb neuronal migration, we performed *ex vivo* cortical electroporation of the full-length neogenin or a control IRES-GFP vector into the ventral telencephalon of wild type E15.5 embryonic brains (Hand et al., 2005). Following electroporation, brains were cultured as slices for 72 hrs to observe migration. Expression of the plasmid carrying GFP positive cells was first observed 24 hrs post-electroporation and subsequently at 72 hrs. At 24 hrs, brains electroporated with either control or neogenin containing plasmids displayed GFP positive cells lining the ventricular zone of the ventral forebrain, with no difference observed between controls and neogenin electroporated cells (Figure 2.6A red). At 72hrs, numerous GFP positive cells from control slices were observed to have migrated considerably from their initial position within the ventricular zone. GFP positive cells from neogenin slices, however, remained clustered within a similar band along the ventricular zone (Figure 2.6A green). Migration was quantified by measuring the total area occupied by GFP positive cells at the endpoint, then

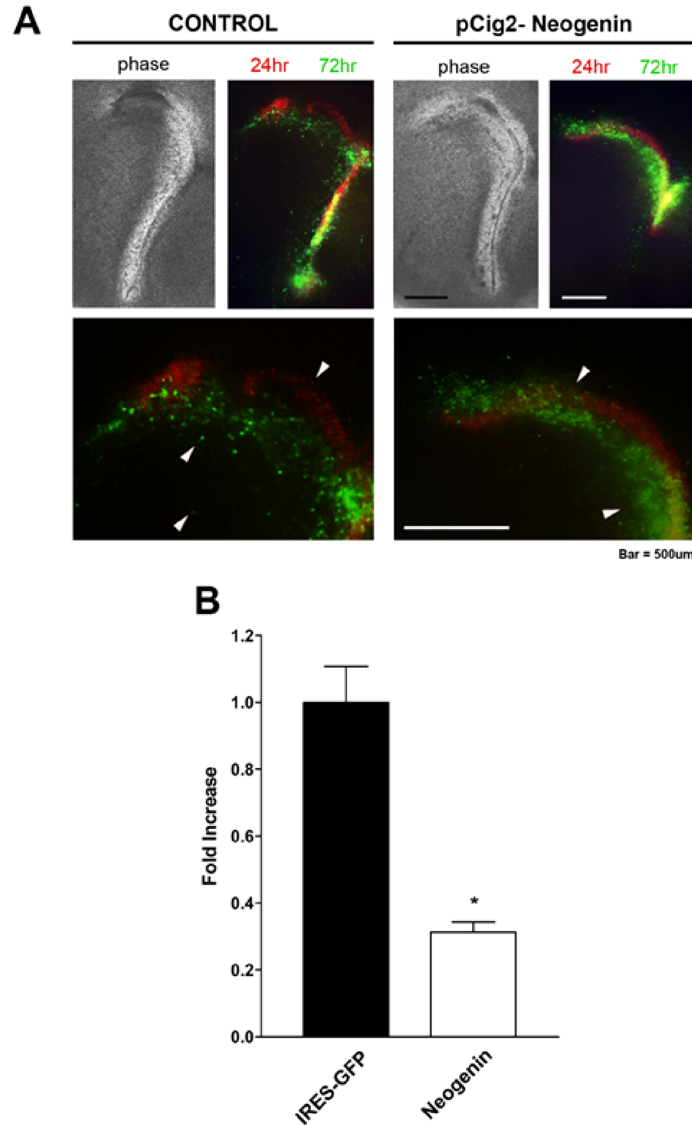

Figure 2.6: **Increased neogenin expression impairs migration of neuroblasts from the subventricular zone.** A) *Ex vivo* overexpression of control IRES-GFP or neogenin-IRES-GFP expressing plasmids in E15 embryos. Embryos were sectioned at 250 µm and plated on poly-L-lysine/laminin coated inserts. Cells were visualized 24 hr post-electroporation (red) to determine their baseline vector expression and migration. Cells were imaged again 72 hr post-electroporation to assess the degree to which they migrated (green). In both control and neogenin overexpressing conditions cells expressing the plasmids initially line the ventricle (red, arrows). After migration the cells move away from the ventricular zone into the striatum (green, arrows) in the control; however, in the mutant they fail to shift position. B) Quantification of the capacity of cells to migrate depicted in A). In order to quantify migration, fold increase was obtained by calculating (Total migration – Initial migration)/Total migration and then normalizing to wild-type migration levels. Error bars represent standard error of the mean (SEM). N = 5 control and N = 6 mutant with 2 sections per embryo. Significance was determined through a paired two-tailed T test of control versus Neogenin overexpressing slices * $p < 0.05$.

subtracting the initial area and dividing this value by the initial area to obtain a percentage increase in migration ((Total migration – Initial migration)/Total migration) and normalized to percentage of control. Upregulation of neogenin resulted in a 77% decrease in migration ($p < 0.05$) compared to that of control electroporated embryos (Figure 2.6B). We conclude that increased expression of neogenin by cells in the ganglionic eminence results in reduced migration of precursors away from the ventricular zone, paralleling the migration defect observed in the Rb deficient forebrain.

Taken together, our results demonstrate a function for the Rb pathway in regulating expression of a non traditional E2F target gene, neogenin, during neuronal migration. Furthermore, we demonstrate that aberrant neogenin expression, similar to that found in conditional Rb mutants, leads to impaired migration. Overall these finding support the conclusion that Rb/E2F regulation of *neogenin* expression, an atypical target, influences appropriate neural development in a manner outside of cell cycle regulation.

DISCUSSION

Here we demonstrate the existence of an Rb/E2F mediated molecular mechanism regulating expression of an atypical E2F target gene, *neogenin*. First we have shown that *neogenin* expression is deregulated in the absence of Rb at the mRNA and protein levels in neural precursor cells. While neogenin has previously been shown to be an E2F regulated target gene *in vitro*, here we complement previous findings by demonstrating that E2F3 is capable of activating neogenin expression and extend them by demonstrating the binding of E2F3 to the 5' regulatory region of the neogenin promoter in neural precursor cells. It is possible that other E2Fs are contributing to regulation of neogenin expression in different biological contexts; however, given that E2F3 has previously been implicated in multiple aspects of nervous system development *in vivo* (Chen et al., 2007; McClellan et al., 2007; McClellan et al., 2009), these observations lend further support that E2F3 regulation is significant in this context. Finally, we demonstrate that E2F transcriptional regulation of neogenin is in turn strongly repressed by Rb activity. These results, along with our data regarding increased neogenin expression among migrating neurons in the absence of Rb suggest a direct role for the regulation of neogenin by the Rb/E2F pathway in the developing forebrain.

Previously we have shown that the Rb/E2F pathway mediates migration of a population of precursors from the ventral telencephalon during nervous system development (Ferguson et al., 2005; McClellan et al., 2007). Here we provide mechanistic insight into this process showing that in the absence of Rb, migrating ventral precursors exhibit a decreased response to the neogenin ligand, netrin-1. Consistent with decreased migration, we observe increased adhesion of ventrally derived Rb deficient precursors to substrate

bound netrin-1. This suggests a mechanism in which increased neogenin expression results in augmented neuronal adhesion leading to the decreased migration. Our *ex vivo* manipulation of neogenin expression resulted in a defect in neuronal migration similar to that seen in the conditional Rb mutant (Ferguson et al., 2005). While we favour our model as a hypothesis to explain how Rb/E2F regulates migration, we note that neogenin is likely only one of many factors contributing to Rb mediated neuronal migration. Indeed neuronal migration is a complex phenomenon involving multiple genes and genetic pathways (Huang, 2009). Through our previous microarray analysis we identified several known genes that regulate migration in the central nervous system and therefore dysfunction in their expression could also be contributing to several facets of the observed migration defect in the conditional Rb mutants (McClellan et al., 2007). While the extent to which deregulated neogenin contributes to the migration defect in Rb mutants is unknown, our studies reveal that overexpression of neogenin perturbs neuroblast migration in wild type tissue (Figure 2.6). Indeed, a rescue experiment in our conditional Rb mutants would be challenging as reducing neogenin expression to physiological levels without causing a complete knock-down would likely lead to variable results. As presented, our results provide strong evidence that, by regulating neogenin expression, Rb/E2F have an important physiological role beyond regulation of the cell cycle machinery, a phenomenon that has not yet been reported. It is probable that Rb is involved in the regulation of multiple genes, which through distinct mechanisms contribute to the regulation of neuronal migration.

The idea that the Rb/E2F pathway can regulate genes outside of the prototypical cell cycle machinery in the context of nervous system development may also broaden its role in tumourigenesis. As the first identified tumour suppressor, intense interest has been focused

on defining the molecular mechanisms through which Rb mediates tumour suppression. While early studies established the model that Rb mediated tumour suppression is the result of its restraint of E2F transcription factors at the G_1/S transition, (reviewed in Weinberg, 1995), more recent studies suggest the role of Rb as a tumor suppressor is more complex than originally hypothesized. Indeed roles for Rb in maintaining genome stability and promoting senescence have broadened the scope and complexity of Rb mediated tumour suppression (reviewed in Goodrich, 2006; Liu et al., 2004). Further deregulation of the Rb pathway in cancer has been traditionally associated with sustained proliferation, however, Rb mutations are frequently found in metastatic cancers including small cell lung carcinoma and osteosarcoma, as well as invasive poor prognosis glioblastomas (reviewed in Classon and Harlow, 2002).

Having demonstrated a novel role for the Rb/E2F pathway in mediating expression of a specific gene involved in neuronal migration, the data presented here raises the possibility that Rb activity could contribute to the regulation of other cellular processes involved in cancer beyond regulation of cell division. Recent studies employing conditional transgenic alleles to remove tumour suppressor genes specifically in adult neural precursor cells have shown the important non-overlapping roles for Rb, PTEN, Nf1 and p53 (Alcantara Llaguno et al., 2009; Jacques et al.). The latter study correlated the ablation of Rb gene expression in the adult subventricular zone with the appearance of primitive neuroectodermal tumours (pNETS). These tumours display significant differentiation across all three neural lineages and ectopic infiltration of surrounding brain tissue. This lends itself to the hypothesis that Rb may also regulate the differentiation and localization of these tumour cells. Many families of genes which mediate neuronal migration, such as the netrin

signalling axis (Fitamant et al., 2008; Rodrigues et al., 2007) have been implicated in multiple aspects of cancer and tumorigenesis. Ligands and receptors from these migration pathways are frequently found deregulated or lost altogether in numerous cancers (reviewed in Arakawa, 2004; Chedotal et al., 2005). Our findings demonstrate a key role for Rb/E2F regulated expression of neogenin. Contributing to neuronal migration gives rise to the possibility that Rb mediated mechanisms may regulate expression of migration related genes during steady-state events such as neurogenesis. The deregulation of these processes may contribute to facets of tumour progression that expand from the typical aberrant S-phase entry associated with Rb loss of function. Further exploration of this hypothesis in the context of tumourigenesis could lend new insight into our understanding of the mechanisms of Rb mediated tumour suppression.

In conclusion, our results suggest that Rb/E2F is required for the regulation of neogenin during neuronal migration. Further these results provide strong support to our overall hypothesis that Rb acts through E2F to mediate events distinct from cell cycle progression by regulating transcription of genes that are not classical E2F targets

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REFERENCES

As per the guidelines "Writing a M.Sc. or Ph.D thesis" from the Department of Cellular and Molecular Medicine at the University of Ottawa, Sub-section "Format of a Collection of Articles", references of all sections are listed in an amalgamated list at the end of the thesis.

http://www.intermed.med.uottawa.ca/cellmed/eng/writing.html

CHAPTER 3

Andrusiak MG*, Vandenbosch R*, Park DS, Slack RS. The retinoblastoma protein is essential to maintain survival of post-mitotic neurons. *J Neurosci*. 2012 Oct 17;32(42):14809-14. doi: 10.1523/JNEUROSCI.1912-12.2012.

*Authors contributed equally to this work

The experiments were conceptualized and performed by MGA and RV. Specifically, RV performed the mouse husbandry and *in vivo* immunohistochemistry. The manuscript was written by MGA and RV. All authors contributed to critical review of the manuscript. RSS and DSP as the principal investigators provided conceptual, technical, and editorial guidance.

Title: THE RETINOBLASTOMA PROTEIN IS ESSENTIAL FOR SURVIVAL OF POST-MITOTIC NEURONS

Abbreviated title: Rb regulates neuronal survival

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Author Contributions: M.G.A, R.V, R.S.S designed research. M.G.A and R.V performed research. M.G.A, R.V, D.S.P, R.S.S analyzed data. M.G.A, R.V, D.S.P and R.S.S wrote the manuscript.

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Abstract

The retinoblastoma protein (Rb) family members are essential regulators of cell cycle progression, principally through regulation of the E2f transcription factors. Growing evidence indicates that abnormal cell cycle signals can participate in neuronal death. In this regard, the role of Rb (p105) itself has been controversial. Germline Rb deletion leads to massive neuronal loss, but initial reports argue that death is non-cell autonomous. To more definitively resolve this question, we generated acute murine knockout models of Rb in terminally differentiated neurons *in vitro* and *in vivo*. Surprisingly, we report that acute inactivation of Rb in post-mitotic neurons results in ectopic cell cycle protein expression and neuronal loss without concurrent induction of classical E2f mediated apoptotic genes, such as Apaf1 or Puma. These results suggest that terminally differentiated neurons require Rb for continuous cell cycle repression and survival.

Keywords: Neurodegeneration, Neuronal quiescence, Cell Cycle

Introduction

The re-expression of cell cycle proteins in neurons, or cell cycle re-entry, has been observed in neurodegenerative conditions and injury such as stroke (Greene et al., 2007; Herrup and Yang, 2007; Rashidian et al., 2007). Recent experimental models implicate essential cell cycle regulators as critical upstream events in disease progression (Park et al., 1997a; Rashidian et al., 2005). For example, Cyclin-dependent kinases (CDKs), key proteins involved in cell cycle progression, have been shown to regulate upstream events during neuronal loss in both *in vitro* and *in vivo* systems (Park et al., 1997a; Rashidian et al., 2005). In these cases, the pathogenic mechanism relating to reactivation of the cell cycle machinery has yet to be fully determined. The paradoxical case of cell cycle reactivation in a seemingly terminally quiescent neuronal population suggests the need for constant repressive action on cell cycle components.

The retinoblastoma protein (Rb), the first tumor suppressor identified, is best characterized for its role in regulating cell proliferation by the repression of E2f transcription factors (Burkhart and Sage, 2008). Upon proliferation-inducing stimulus, Rb is phosphorylated by CDK-Cyclin complexes which disassociate the Rb/E2f complex allowing for the transcription of genes that initiate S-phase of the cell cycle (Burkhart and Sage, 2008). Rb has also been shown to regulate apoptosis by both direct and indirect mechanisms (Polager and Ginsberg, 2009). Rb targets include genes regulating cell cycle progression (CyclinE1, CyclinA2) (Burkhart and Sage, 2008), apoptosis (Apaf1, Puma, SIVA) (Polager and Ginsberg, 2009), and non-classical functions (Neogenin, Bnip3) (Andrusiak et al., 2011; Tracy et al., 2007). Previous studies examining the role of cell-cycle regulators in neuronal cell death observed that CDK inhibition, as well as Rb overexpression are protective against

apoptotic stimuli (Park et al., 2000; Park et al., 1997b). Additionally, Rb is phosphorylated in a number of neuronal injury and degeneration models (Biswas et al., 2007; Park et al., 2000; Yu et al., 2012). These studies initially suggested that CDK mediated inactivation of Rb may be critical in mediating neuronal loss; however, several pieces of evidence questioned this interpretation. First, while Rb germline knockout mice showed massive neuronal loss, this was due to placental defects rather than neuron specific pathways (Clarke et al., 1992; de Bruin et al., 2003; Jacks et al., 1992; Lee et al., 1992). Consistent with this, conditional Rb-deficient neural precursors divided ectopically, but were able to survive and differentiate into neurons (Ferguson et al., 2002; MacPherson et al., 2003). Whether this was due to compensation by the Rb homologs p107 and p130 is unknown. Second, previous studies implicated p130 and not Rb as the main mediator of neuronal death particularly in models of nerve growth factor (NGF) withdrawal (Liu et al., 2005). Taken together it poses the important question of whether Rb itself plays any significant role in neuronal cell cycle mediated death.

Here, we sought to define the specific role for Rb in terminally differentiated post-mitotic neurons. We show that Rb is essential to maintain survival of post-mitotic neurons throughout adulthood. Rb loss induces expression of cell cycle regulatory genes; however, death following Rb loss appears to occur independently of transcriptional up-regulation of apoptotic genes. This evidence demonstrates a crucial role for Rb in maintaining quiescence and survival of post-mitotic neurons.

Materials and Methods

Animals

All experiments were approved by the University of Ottawa's Animal Care Ethics Committee adhering to the Guidelines of the Canadian Council on Animal Care. Rb^{flox} (Marino et al., 2000) and CamKCreERT2 (EMMA ID: 02125) (Erdmann et al., 2007) mice were maintained on FVBN and C57Bl/6 backgrounds, respectively. Animals were genotyped according to standard protocols with previously published primers. All CamKCreERT2 animals used were heterozygotes for Cre expression. CamKCreERT2;$Rb^{flox/+}$ mice were crossed with $Rb^{flox/flox}$ mice to generate experimental animals, which at 5-6 weeks of age, were given tamoxifen (Sigma) (180 mg/kg/day i.p., 5 days) and sacrificed one or four weeks after the final injection. In all experiments both female and male animals were used.

Primary Cortical Neurons

Embryonic cortical neurons were isolated by standard procedures (Fortin et al., 2001). Neurons were infected at the time of plating with a pWPXLD lentiviral vector expressing either control GFP, GFP-tagged Cre recombinase or dominant negative $DP1_{\Delta 103\text{-}126}$ at a multiplicity of infection (MOI) of 2. For immunofluorescence, cells were grown on coverslips and treated as indicated in the figure legends. Cells were then fixed with 4% paraformaldehyde (PFA) and stained for Tuj1 (Covance), Ki67 (Cell Marque), γ-H2AX (Millipore) and DAPI. AlexaFluor 488 /594 secondary antibodies were used (Molecular Probes). Statistical differences were determined using a one-way ANOVA, where $p<0.05$ was considered statistically significant.

Western Blots

Protein was isolated from cultured cortical neurons or cortical tissue and western blot analyses performed as previously described (Ferguson et al. 2000) with antibodies directed towards activated caspase-3 (Cell Signaling), Rb (Pharmingen), CyclinA2 (Abcam), γ-H2AX (Millipore), and β-actin (Sigma).

Real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) assays were performed on primary cortical neurons using a Rotor-Gene RG-3000 (Corbett research, Sydney, Australia). Total RNA was isolated using Trizol® method (Invitrogen, Carlsbad, CA, USA) at 6 DIV. The SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) was used to amplify indicated target genes. All expression values were normalized to GAPDH. Primer sequences are available upon request. Expression values were obtained from three independent cultures and significance was determined by a two-tailed, Student's *t*-test, where $p<0.05$ was considered statistically significant.

Microarray

$Rb^{flox/flox}$ neurons were treated with GFP or Cre-GFP lentivirus and harvested after 6 days *in vitro* from three independent dissections. Total RNA was purified using Trizol® method (Invitrogen, Carlsbad, CA, USA). Samples were hybridized to Mouse Gene 1.0ST array (Affymetrix) at the Ottawa Hospital Research Institute Stemcore facility (Ontario, Canada). Data was normalized using the Robust Multichip Average (RMA) expression measure (Irizarry et al., 2003), and statistically significant gene changes were determined using significance analysis of microrarrays (Tusher et al., 2001) in the TM4 MultiExperiment

Viewer software package (Saeed et al., 2003). Significant changes in gene expression were computed using a fold-increase of ≥1.5 and a false discovery rate (FDR) of <5%. Gene functional classification was performed using DAVID Bioinformatics (http://david.abcc.ncifcrf.gov/).

Tissue processing, immunohistochemistry and cell quantification

Brains were perfused and fixed as previously described (Fortin et al., 2001). Sections were collected as 14-µm coronal cryosections on slides. For immunohistochemistry, sections underwent antigen retrieval in Target Retrieval Solution (Dako, Glostrup, Denmark) and incubated overnight at 4°C with the following primary antibodies: NeuN (Millipore), Ki67 (Cell Marque) and γ-H2AX (Millipore). Sections were incubated in blocking solution containing donkey anti-rabbit AlexaFluor488 (Invitrogen) or donkey anti-mouse Cy3 (Jackson Immunoresearch) and DAPI. All images were acquired using a Zeiss 510 metaconfocal microscope under a 40X objective. For cell quantification, a minimum of 3 sections containing the frontal cortex was analysed per brain. For each section, two fields in layer 2-4 and two fields layer 5/6 were picked in the primary motor cortex, and the percentage of NeuN+ cells among the total DAPI+ cells, Ki67+ cells among NeuN+ cells or γ-H2AX+ cells among Ki67+ cells were quantified. Statistical analysis was determined by a two-tailed, Student's *t*-test, where $p<0.05$ was considered statistically significant.

Results

Rb loss triggers apoptosis in primary cortical neurons

Previous evidence as stated above suggested that Rb does not regulate cell death in immature neural precursors. We were interested to see whether Rb may play a role in fully differentiated neurons. To accomplish this, we first established an acute paradigm in which we treated primary cortical neurons from animals harboring a conditional Rb allele ($Rb^{flox/flox}$) with lentiviruses expressing control-GFP (GFP) or Cre-GFP (Cre). Lentivirus carrying Cre was tested and had no toxic effects in wild type neurons (Appendix A). This system ablates Rb in cells infected with Cre virus (Figure 3.1B). We examined neurons for induction of apoptosis by both microscopic assessment of condensed nuclei and western blot analysis for activated caspase-3 (AC-3) (Figure 3.1A-B). Rb loss was observed as early as 4 days after viral infection of Cre. Analysis of condensed nuclei by DAPI staining showed very little effect in Cre infected neurons at 4 days *in vitro* (DIV); however, robust death was observed by 10DIV (Figure 3.1A). Activation of caspase-3 by western blot analysis revealed an identical trend with caspase-3 activation peaking slightly earlier at 8 DIV (Figure 3.1B). This evidence supports the notion that Rb plays an essential role in the survival of post-mitotic neurons. To address whether the neuronal apoptosis we observe is E2f-dependent, we broadly inhibited E2f activity using a dominant negative $DP1_{\Delta 103\text{-}126}$ construct (Wu et al., 1996). The inhibition of E2f activity was able to rescue the appearance of condensed nuclei upon Rb-deletion, and had no deleterious effect on control neurons (Figure 3.1C).

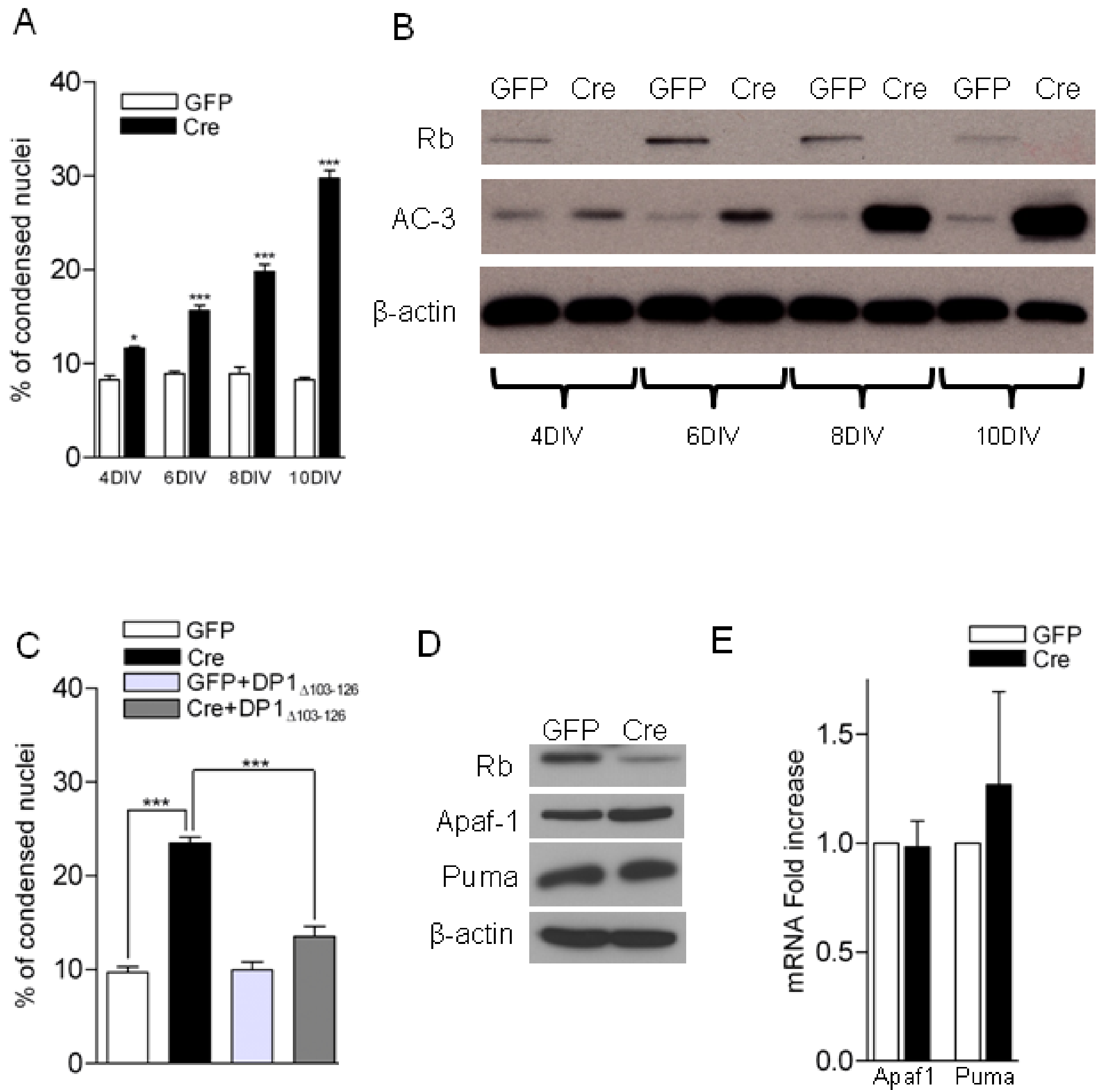

Figure 3.1. *Acute Rb removal results in neuronal apoptosis independent of classical E2f regulated apoptotic genes.* A) Cortical neurons were fixed at the indicated days *in vitro* (DIV) and condensed nuclei were examined by DAPI staining (n=3). B) Western blot analysis of total protein extracted from Control and Rb-deficient cortical neurons at the indicated DIV. C) Cortical neurons were infected with the indicated constructs and condensed nuclei were examined by DAPI staining at 8DIV (n=3). D) Western blots on total protein extracted at 6DIV. E) qRT-PCR on RNA extracted at 6DIV. RNA levels were normalized to GAPDH (n=3). $*p<0.05$, $***p<0.001$.

Rb loss does not induce classical E2f-regulated apoptotic genes

The Rb/E2f axis has been implicated in the direct transcriptional regulation of apoptotic machinery in a number of models (Polager and Ginsberg, 2009). The most well characterized gene targets consist of Apaf1 and the BH3-only protein Puma (Guo et al., 2001; Hershko and Ginsberg, 2004). We therefore asked the question if loss of Rb de-represses these apoptotic genes thereby triggering a cell death cascade. We examined levels of the E2f-regulated apoptotic genes Apaf1 and Puma by western blot and qRT-PCR (Figure 3.1D-E). We did not observe any significant up-regulation of either gene at the level of protein or transcript suggesting an alternative pathway of death.

Acute removal of Rb results in up-regulation of cell cycle machinery

We next sought to identify the specific genes de-repressed upon loss of Rb in our primary cortical neurons. We employed microarray to examine broad gene expression changes in response to Rb deletion. We examined neurons at 6DIV as they do not yet display extensive apoptosis which may result in indirect transcript dysregulation. Genes were deemed significant by significance analysis of microarray (SAM) if increased ≥1.5 fold and a false discovery rate (FDR) of ≤5% (n=3). We identified 377 increased transcripts upon Rb loss (Figure 3.2A). Using DAVID Bioinformatics (http://david.abcc.ncifcrf.gov/) we employed a functional classification of genes (Huang da et al., 2009) (Figure 3.2B). Gene functional classification utilizes a modular enrichment analysis (MEA) to assess term-to-term relationships in order to minimize redundancy in GO classification terms. Of the 377 genes identified as up-regulated in our Rb-deficient neurons, 368 possessed DAVID IDs. Gene functional classification identified 14 gene clusters representing 216 genes. These

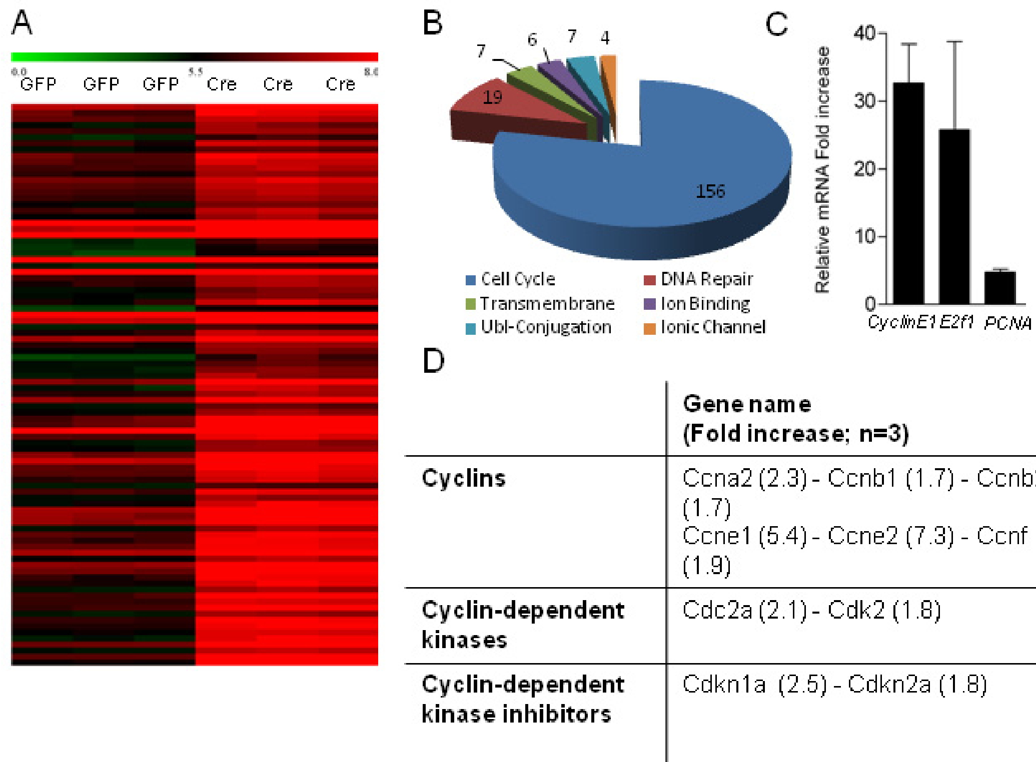

Figure 3.2. *Loss of Rb up-regulates genes associated with cell cycle function.* A) Heat map representing top 200 genes significantly up-regulated by microarray upon acute Rb deletion (fold-increase ≥1.5, FDR <5%, n=3). B) Gene functional classification of up-regulated transcripts following Rb loss. C) qRT-PCR validation of targets identified by microarray analysis. D) Analysis of distribution of cell cycle regulatory genes induced upon Rb-deletion.

clusters were manually reduced to Cell Cycle (156), DNA Repair (19), Transmembrane (7), Ion Binding (6), Ubl-conjugation (7) and Ionic Channel (4) (Figure 3.2B). This over-representation of cell cycle genes suggests a restricted role for Rb in post-mitotic neurons in the maintenance of the quiescent state. Several genes including PCNA and CyclinE1 were chosen for validation by qRT-PCR with up-regulation on the array confirmed (Figure 3.2C). Up-regulated cell cycle genes were not confined to the G1/S transition, as factors associated with various facets of the cell cycle were induced (Figure 3.2D). Our microarray data suggests that Rb plays a role in the regulation of specific gene classes that does not include classical E2f-regulated apoptotic genes in terminally differentiated neurons.

Acute removal of Rb triggers ectopic cell cycle protein expression and DNA damage

Our microarray analysis revealed a very specific cohort of genes related to cell cycle dynamics up-regulated in the absence of Rb. We then asked whether up-regulation of these genes would manifest itself as ectopic expression of cell cycle proteins. $Rb^{flox/flox}$ neurons infected with control and Cre lentivirus were fixed and stained for Tuj1 (neuronal marker) and Ki67 (proliferative marker). We observed Tuj1/Ki67+ very prominently in Rb-deficient neurons, which were rarely visible in control neurons (Figure 3.3A-B). Neurons also exhibited an increase in protein levels of the known cell cycle regulatory protein CyclinA2 (Figure 3.3C). Finally, we examined neurons for increased expression of γ-H2AX. γ-H2AX is the phosphorylated form of H2AX; this phosphorylation event is dependent upon induction of double strand breaks (DSB) (Rogakou et al., 1998). We observed a significant increase in this mark in Rb-deficient neurons (Figure 3.3C), suggesting that Rb loss triggers ectopic induction of cell cycle machinery and a DNA damage response. The induction of

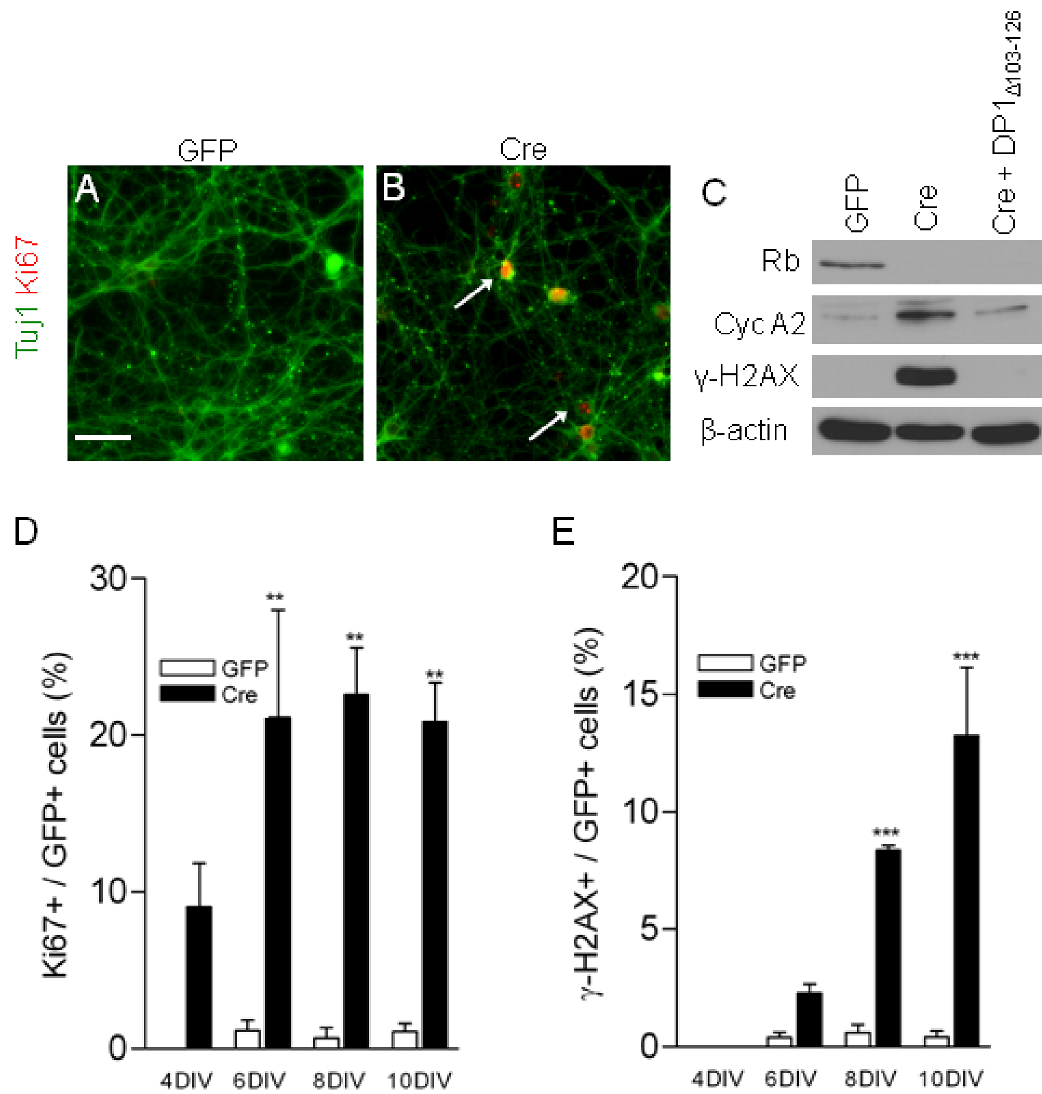

Figure 3.3. *Rb-deficient neurons display cell cycle and DNA damage markers.* A-B) Cortical neurons were fixed at 8DIV and stained for Ki67. C) Western blots were performed on total protein extracted at 8DIV. D-E) Cortical neurons were fixed at the indicated DIV and quantified for the percentage of double labelling (Ki67+/GFP+ over GFP+, γ-H2AX/GFP+ over GFP+)(n=3 or 4). **p<0.01,***p<0.001._Scale bar = 50μm.

γ-H2AX and CyclinA2 is E2f-dependent as introduction of $DP1_{\Delta 103\text{-}126}$ was able to rescue to control levels (Figure 3.3C). To examine the temporal relationship between cell cycle re-entry and DNA damage, we immunostained for Ki67 and γ-H2AX at 4, 6, 8 and 10DIV. We observed an increase in the number of Ki67+ cells upon Rb deletion as early as 4DIV (Figure 3.3D). The appearance of γ-H2AX was not apparent until 6DIV and increased at 8 and 10DIV (Figure 3.3E). This data suggests that double strand breaks occur downstream of cell cycle re-entry and that both of these processes are mediated by E2f-activation.

Acute in vivo loss of Rb results in neurodegeneration

We finally asked whether conditional Rb loss would result in neurodegeneration of adult neurons *in vivo*. To this end, we employed a tamoxifen (TAM)-inducible CamKCreERT2 mouse model to acutely delete Rb in adult forebrain neurons. Adult CamKCreERT2; $Rb^{flox/+}$ (Ctrl) and CamKCreERT2; $Rb^{flox/flox}$ mice (5-6 weeks of age) were injected with TAM and sacrificed one or four weeks after the last injection (Figure 3.4A). Recombination efficiency was validated by western blot (Figure 3.4B). At one week post-injection, we did not detect any significant neuronal loss in the cerebral cortex of CamKCreERT2; $Rb^{flox/flox}$ animals (Figure 3.4C). However, neuronal density was dramatically reduced in the cortex of knockout animals four weeks after TAM induction (Figure 3.4C-E). To determine whether Rb-deficient neurons ectopically express cell cycle proteins *in vivo*, we co-labelled the neuronal marker NeuN with Ki67 at one week post-injection, prior to neuronal loss. We did not observe NeuN/Ki67+ neurons in Ctrl animals, however; 22.5±16.2% and 16.9±3.9% (n=3) of NeuN+ neurons expressed Ki67 in the superficial (2-4) and deep (5/6) layers of Rb-deficient animals, respectively (Figure 3.4F-G).

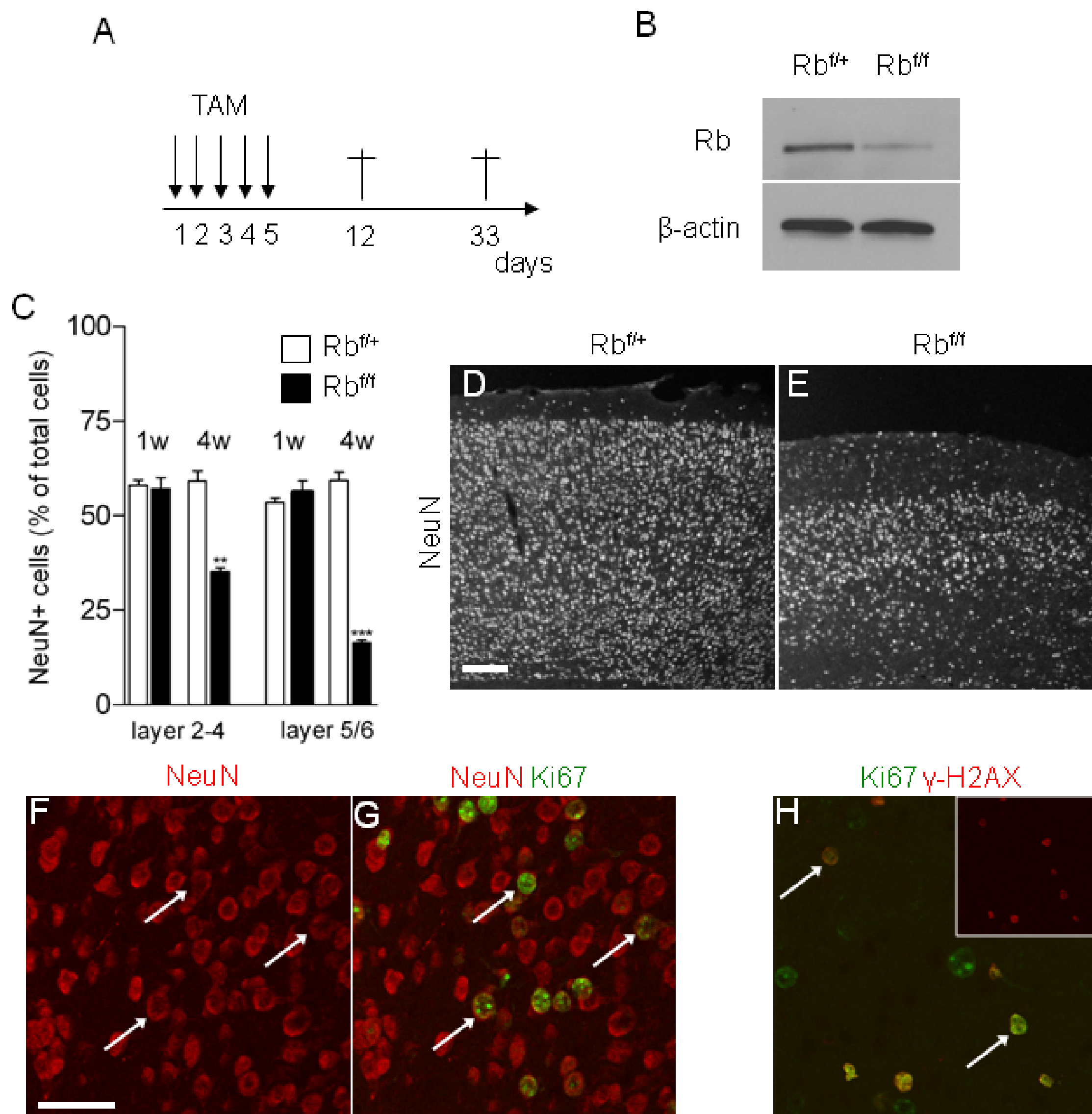

Figure 3.4. *Acute Rb removal in adult neurons triggers neuronal loss.* A) Tamoxifen (TAM) injection paradigm. B) Western blot of cortex lysates confirming the decrease of Rb levels in CamKCreERT2; $Rb^{flox/flox}$mice ($Rb^{f/f}$) one week after the last TAM injection. **C)** Rb loss results in neuronal loss in the cerebral cortex four weeks following TAM treatment (n=3). D-E) Representative pictures of NeuN immunofluorescence displaying loss of neurons in the cortex of CamKCreERT2; $Rb^{flox/flox}$mice (E) compared to CamKCreERT2; $Rb^{flox/+}$mice (D). F-G) Ectopic expression of the proliferative marker Ki67 in NeuN+ neurons (arrows) of CamKCreERT2; $Rb^{flox/flox}$ mice one week after the last TAM injection. H) Ectopically proliferating Ki67+ cells exhibit γ-H2AX immunoreactivity (arrows) following Rb deficiency. **$p<0.01$,***$p<0.001$. Scale bar = 100μm (D,E) and 50μm (F-H).

Finally, a significant proportion of ectopic Ki67+ cells in the cortex of Rb-deficient animals also exhibit γ-H2AX immunoreactivity (layer 2-4: 81.2±7.3%; layer 5/6: 54.9±5.1%; n=3), indicating that Rb loss induces cell cycle proteins and DSB, which may contribute to neuronal loss *in vivo* (Figure 3.4H).

Discussion

Our studies demonstrate several significant conclusions regarding neuronal survival. We have shown that acute loss of the cell-cycle regulatory gene Rb in post-mitotic neurons results in apoptosis. Critically, we show by microarray and candidate gene approaches that this neuronal loss is not due to de-repression of classical Rb/E2f regulated apoptotic machinery. Instead, we observe the induction of mainly cell cycle and DNA repair related proteins. Finally, we provide *in vivo* evidence that acute Rb deletion is sufficient to induce neuronal loss. Taken together, these results highlight a specific role for Rb in the repression of cell cycle genes and survival in post-mitotic neurons.

Importantly, our results highlight a differential dependence on Rb in proliferating neural precursors and terminally differentiated neurons. Germline Rb loss models displayed non-autonomous neuronal apoptosis, and *in vitro* differentiation of germline Rb-deficient precursors showcased the dispensable role for Rb at this stage of development (Callaghan et al., 1999). In more defined precursor-specific conditional deletion models, neuronal differentiation also proceeded in a largely normal fashion (Ferguson et al., 2002; MacPherson et al., 2003). In contrast, our present observations indicate that in terminally differentiated neurons, Rb is essential to maintain survival.

Our findings serve to further expand the role of the Rb family in the regulation of neuronal survival. In both steady-state and stress-induced paradigms, the Rb-related protein p130 was shown to be essential in the regulation of neuronal survival (Liu et al., 2005). Our data implicates Rb in a similar manner. It is important to note the latencies to cell death observed with either p130 or Rb loss. Upon siRNA mediated p130 depletion, apoptosis was

observed within 48h (Liu et al., 2005) whereas, in our system, Rb deletion results in similar levels of cell death after 120h. This suggests that while both Rb and p130 may be essential in the maintenance of neuronal survival, the mechanisms employed by each may differ. In neurons, p130 was shown to mediate gene repression via HDAC1 mediated interaction with Suv39h1-p130-E2f4 (Liu et al., 2005). The direct mechanisms that Rb employs to repress gene expression in neurons has yet to be fully described.

We and others have previously demonstrated a tight link between neuronal cell cycle re-entry and cell death. Importantly, our data emphasize that high E2f activity and induction of E2f target genes in the absence of Rb is deleterious for post-mitotic neurons. However, the direct link between cell cycle re-entry and cell death remains correlative at this point, and future experiments will be required to determine exactly how cell cycle re-entry and DNA damage repair intersect to result in cell death in a post-mitotic neuron. To our surprise, induction of cell death upon Rb deletion occurs independently of E2f dependent transcriptional up-regulation of classical Rb/E2f-mediated apoptotic factors such as Apaf1 and Puma. In this regard, comparison to other neuronal death paradigms involving abnormal cell cycle activation is informative. DNA damage-induced CDK activation, Rb phosphorylation and subsequent cell loss occurs within hours of insult (Park et al., 2000). However, in this paradigm, independent activation of both CDK/Rb/E2f and p53 signals occur and work in concert to promote apoptosis (Morris et al., 2001). In contrast, our present data indicates that Rb deficiency alone results in a significantly protracted timing of death in comparison to DNA damage. This suggests that the efficiency and speed of death is regulated by the presence of multiple signals which act together to regulate apoptosis. In this regard, classic apoptotic targets like Apaf1 and Puma are known to be regulated by both

E2fs and p53. Finally, it is interesting to note that while Rb loss itself induces markers of DNA damage and induction of DNA repair enzymes, the classic p53 response does not appear to be initially activated.

In conclusion, our results reveal an essential role for Rb in the maintenance of neuronal survival. Rb is able to actively repress genes that drive the cell cycle in order to prevent neurons from entering a proliferative state. Unlike other tissue systems, Rb loss does not trigger concurrent induction of classical E2f-regulated apoptotic targets. These findings reveal the need for constitutive Rb mediated repression in order to prevent ectopic expression of cell cycle proteins and maintain survival in a terminally differentiated post-mitotic neuron. It also highlights the differential role Rb plays in precursors compared to terminally differentiated neurons.

REFERENCES

As per the guidelines "Writing a M.Sc. or Ph.D thesis" from the Department of Cellular and Molecular Medicine at the University of Ottawa, Sub-section "Format of a Collection of Articles", references of all sections are listed in an amalgamated list at the end of the thesis.

http://www.intermed.med.uottawa.ca/cellmed/eng/writing.html

CHAPTER 4

Andrusiak MG, Vandenbosch R, Park DS, Dick FA, Slack RS. LXCXE-independent chromatin remodeling by Rb/E2f mediates neuronal quiescence. *Cell Cycle*. 2013 May 1;12(9) *In press*

The experiments were conceptualized and performed by MGA. RV provided technical assistance and helpful discussion to aid in the conceptualization of several experiments. FAD provided the mutant LXCXE *murine* transgenic strain. MGA wrote the manuscript, with all authors providing critical reviews. RSS as the principal investigator provided conceptual, technical, and editorial guidance.

Title: LXCXE-independent chromatin remodeling by Rb/E2f mediates neuronal quiescence

Abbreviated title: LXCXE-independent gene repression

Keywords: Rb, E2f, Neuronal quiescence, Cell Cycle, Transcription

Abbreviations: AC-3, activated caspase-3; Acetyl-H3, acetylated histone 3; CycA2, Cyclin A2; DAPI, 4',6-diamidino-2-phenylindole; DIV, days *in vitro*; DNA, deoxyribonucleic acid; DP1, dimerization partner 1; E1A, early region 1A; GFP, green fluorescent protein; H3K9me3, trimethylated histone 3 on lysine 9; H3K4, histone 3 lysine 4; HDAC, histone deacetylase; LV, lentivirus; LXCXE, leucine- any amino acid- cysteine- any amino acid- glutamate; ΔL, LXCXE-binding disruption mutant; NeuN, neuronal nuclei; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; Rb, retinoblastoma; SV40, simian virus 40.

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Abstract

Neuronal survival is dependent upon the retinoblastoma family members, Rb1 (Rb) and Rb2 (p130). Rb is thought to regulate gene repression, in part, through direct recruitment of chromatin modifying enzymes to its conserved LXCXE binding domain. We sought to examine the mechanisms that Rb employs to mediate cell cycle gene repression in terminally differentiated cortical neurons. Here we report that Rb loss converts chromatin at the promoters of E2f-target genes to an activated state. We established a mouse model system in which Rb-LXCXE interactions could be inducibley disabled. Surprisingly, this had no effect on survival or gene silencing in neuronal quiescence. Absence of the Rb LXCXE-binding domain in neurons is compatible with gene repression and long-term survival, unlike Rb deficiency. Finally, we are able to show that chromatin activation following Rb deletion occurs at the level of E2fs. Blocking E2f mediated transcription, downstream of Rb-loss, is sufficient to maintain chromatin in an inactive state. Taken together our results suggest a model whereby Rb-E2f interactions are sufficient to maintain gene repression irrespective of LXCXE-dependent chromatin remodeling.

Introduction

The retinoblastoma protein (Rb) plays an essential role in regulating cell proliferation by the repression of E2f transcription factors.(Burkhart and Sage, 2008) Rb/E2f interactions are best characterized for their role in regulating the G_1/S transition; however, growing evidence is mounting to suggest a more diverse role for the pathway in both cell cycle and non-cell cycle gene regulation (Burkhart and Sage, 2008). In addition to directly inhibiting transcription through interaction with E2fs, Rb is able to actively repress gene expression through the recruitment of co-factors (Dick, 2007). Rb is thought to mediate many of these repressive effects through its conserved LXCXE binding domain (Dick, 2007).

The study of viral oncoproteins, such as E1A and SV40 Large T-antigen, led to the identification of the conserved LXCXE motif that mediates interactions with Rb (DeCaprio et al., 1988; Wang et al., 1993b). The LXCXE domain was shown to be essential for the inactivation of Rb by direct interaction with oncoproteins (Dyson et al., 1992; Kim et al., 2001). Further studies identified similar LXCXE-interaction domains found in these viral proteins in other cellular constituents (Dick, 2007). Seminal studies showed that factors, such as HDAC1, which contain an LXCXE-interacting domain, interact with Rb and are essential for its repressive function under certain contexts (Magnaghi-Jaulin et al., 1998). Subsequent studies identified LXCXE interacting factors that modulate transcriptional activation by histone methylation and nucleosome remodeling (Dunaief et al., 1994; Nielsen et al., 2001) Other non-transcriptional LXCXE interactions have also been revealed such as cyclin D and BRCA1 which are important for Rb function and tumor suppression (Dowdy et al., 1993; Ewen et al., 1993; Fan et al., 2001). Understanding how these LXCXE

interactions shape Rb/E2f activity physiologically is imperative in our knowledge of how Rb loss contributes to pathological events such as tumorigenesis and neurodegeneration (Burkhart and Sage, 2008; Rashidian et al., 2005).

The development of a *murine* transgenic knock-in mutant in which Rb is defective for LXCXE interactions has challenged initial studies detailing its importance in Rb-mediated gene repression. The *in vivo* observation that LXCXE interactions are important under conditions of stress such as senescence and following DNA damage suggest unique contexts where this domain is required (Bourgo et al., 2011; Isaac et al., 2006; Talluri et al., 2010). Examination of fully differentiated retinal neurons deficient for the LXCXE-binding domain of Rb did not reveal any proliferative or differentiation defects (Talluri et al., 2010); however, the use of germline homozygosity may have allowed for compensation by other Rb family members. Furthermore, retinal neurons display a proliferative competence suggesting potential differences in gene-repression when compared to a proliferation-incompetent quiescent cell type such as cortical neurons (Ajioka et al., 2007; Andrusiak et al., 2012; Busser et al., 1998). It is still unknown how Rb mediates long-term gene repression in a population that is incapable of proliferation.

Given our previous observation that Rb is essential for mediating cell cycle gene repression and survival in post-mitotic neurons, we sought to examine the mechanism utilized by Rb to regulate this repression (Andrusiak et al., 2012). Cortical neurons present an excellent system to study quiescence, as they are terminally differentiated cells and should therefore employ a consistent non-transient mechanism to repress cell cycle related transcription. Using our previously established acute model for Rb deletion *in vitro*, we observe an activation of chromatin at E2f-regulated gene promoters following Rb loss. We

next examined the dependence on LXCXE-dependent interactions for gene repression and cell cycle regulation, as this domain has been broadly implicated in Rb-mediated chromatin remodeling. We surprisingly report that Rb-LXCXE interactions are dispensable for long-term regulation of cell cycle gene repression and neuronal survival both *in vitro* and *in vivo*. To address the dependence of chromatin activation on Rb and E2f, we broadly inhibited E2f activation in the absence of Rb. The inhibition of E2f activation was sufficient to prevent chromatin activation, suggesting that this process is dependent on E2f-activation and not Rb-mediated repression in post-mitotic cortical neurons. In this study, we highlight the sufficiency of Rb-E2f interactions in maintaining neuronal quiescence and survival.

Results

Rb loss induces an active chromatin state at E2f target genes

Our previous study had identified an essential role for Rb in the survival of post-mitotic cortical neurons (Andrusiak et al., 2012). We observed specific induction of cell cycle related gene targets and disruption of neuronal quiescence upon acute removal of Rb (Andrusiak et al., 2012). Given the previously described role for Rb in the regulation of chromatin dynamics, we were interested to see the impact of Rb deletion on the chromatin landscape in quiescent cortical neurons.

To ask whether Rb plays a role in regulating chromatin dynamics, $Rb^{Flox/Flox}$ neurons were infected with either GFP or Cre expressing lentiviruses, as previously described (Andrusiak et al., 2012), then fixed and harvested at 6 days *in vitro* (DIV). We performed chromatin immunoprecipitation with antibodies directed towards acetylated histone 3 (Acetyl-H3) and tri-methylated lysine 9 on histone 3 (H3K9me3), as these are two well-

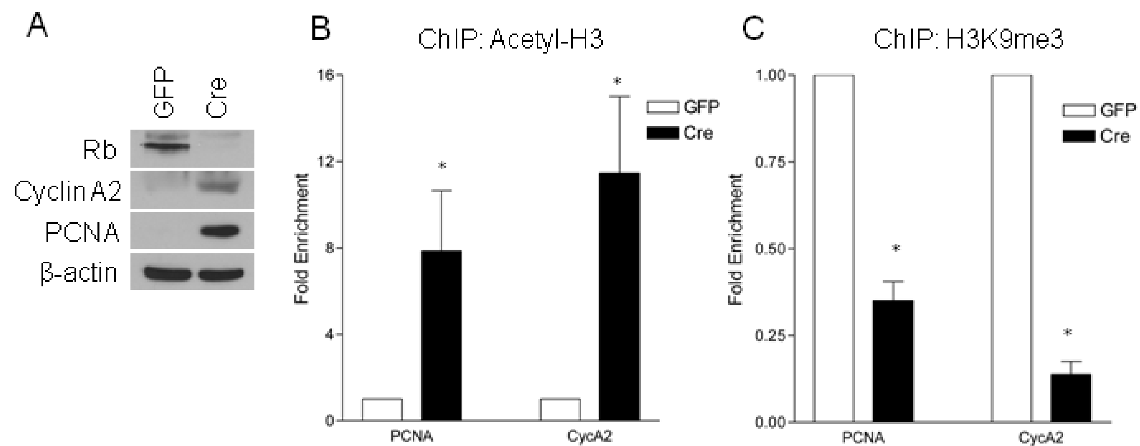

Figure 4.1. *Acute Rb removal results chromatin activation at E2f-regulated promoters.* qRT-PCR analysis of the *PCNA* and *CycA2* promoters in GFP or Cre infected cortical neurons ($Rb^{Flox/Flox}$) at 6DIV from chromatin immunoprecipitated DNA. A) Western blot analysis of total protein extracted from Control (GFP) and Rb-deficient (Cre) cortical neurons at 6DIV. B) Chromatin was immunoprecipitated with an antibody directed towards acetylated histone 3 (Acetyl-H3) C) Chromatin was immunoprecipitated using an antibody that recognizes trimethylated lysine 9 on histone 3 (H3K9me3). Error bars represent SEM (n=3, where n represents a ChIP performed on neurons isolated from a unique embryo), significance was determined using a two-tailed Student's T-test where *$p<0.05$.

characterized Rb-associated chromatin marks (Dick, 2007). Using the *PCNA* and *CyclinA2* promoters as established E2f-regulated gene products (Burkhart and Sage, 2008), we performed quantitative real-time PCR to assess the enrichment of Acetyl-H3 and H3K9me3 at these promoters in the presence or absence of Rb (Figure 4.1B-C). In accordance with an increase at the protein level by western blot (Figure 4.1A), Rb deletion induces the chromatin-activation mark Acetyl-H3 at both the *PCNA* and *CycA2* promoters, and a significant decrease in the repressive H3K9me3 mark (Figure 4.1B-C). This evidence supports the notion that Rb plays a role in the regulation of gene-repression at the level of chromatin in quiescent cortical neurons.

The Rb LXCXE-binding domain is dispensable for neuronal quiescence

We next sought to determine a more specific role for Rb in the regulation of chromatin dynamics in cortical neurons. We focused on the Rb LXCXE binding motif for its previously established role in chromatin regulation by interaction with specific factors involved in histone acetylation and histone methylation (Dick, 2007). Cortical neurons present an excellent model to study the role of Rb in gene repression as they are 1) a proliferation incompetent terminally quiescent cell type and 2) have limited secondary consequences due to their inability to divide. In order to address this question, we established an acute Rb LXCXE-deficiency model ($Rb^{\Delta L}$) (Figure S4.1A). This specific $Rb^{\Delta L}$ mutant has been extensively characterized to be deficient in interacting with known LXCXE-dependent factors (Isaac et al., 2006; Talluri et al., 2010). Our model employs $Rb^{Flox/\Delta L}$ with the addition of Cre, by viral or transgenic methods, inducing acute removal of the floxed allele, leaving the mutant $Rb^{\Delta L}$ protein to function. This model prevents any compensation due to the long-term absence of Rb or the LXCXE binding domain.

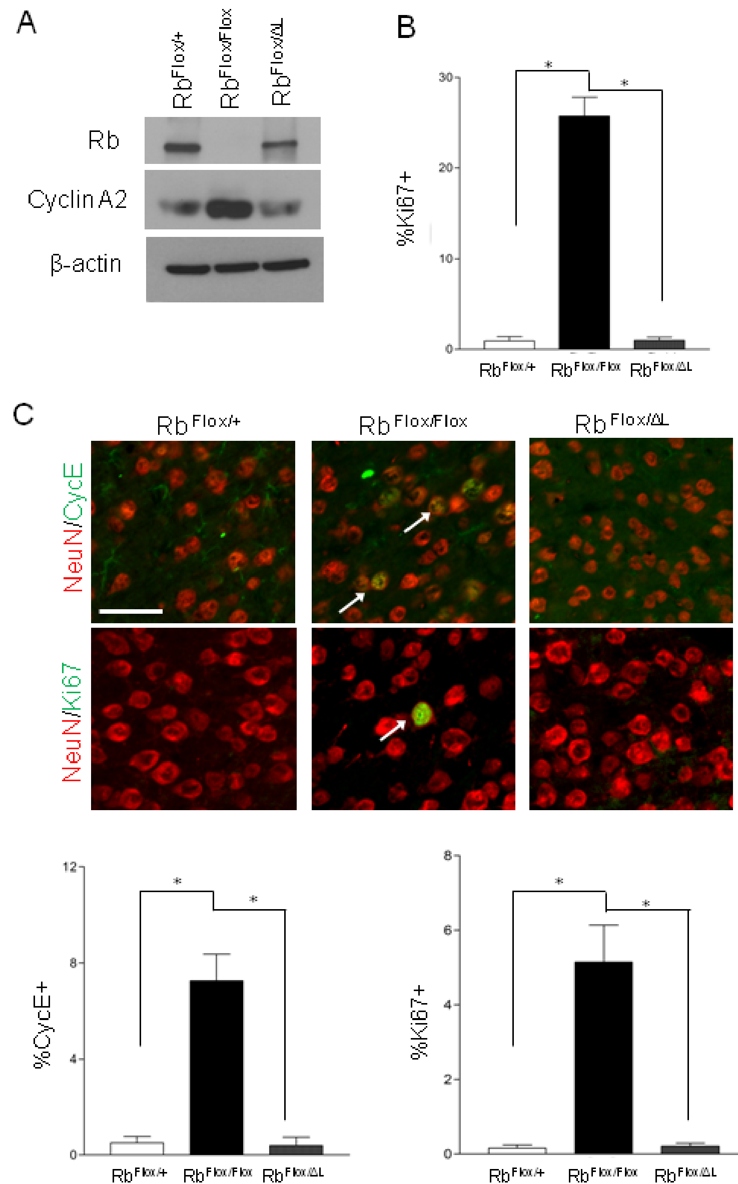

Figure 4.2. *Rb regulates neuronal quiescence in an LXCXE independent manner* A) Western blot analysis of total protein extracted from Control, Rb-deficient and $Rb^{\Delta L}$ cortical neurons at 10DIV B) Cortical neurons were fixed at 10DIV and stained for Ki67. Percentage of Ki67+ was quantified using DAPI to represent total cell number. Error bars represent SEM (n=3, where n represent an independent culture with multiple technical replicates), statistical significance was determined using a one-way ANOVA, where $*p<0.05$ was considered statistically significant. C) Animals of indicated genotypes were injected with tamoxifen (180 mg/kg/day i.p., 3 days) and euthanized four weeks following the final injection. Representative pictures of ectopic expression of the cell-cycle related factors Ki67 (arrows) and Cyclin E (arrows) in neurons in the cortex of CamKCreERT2; $Rb^{Flox/Flox}$mice compared to CamKCreERT2; $Rb^{Flox/+}$ and CamKCreERT2; $Rb^{Flox/\Delta L}$mice. Error bars represent SEM (n=3, where n represent the cortex from a unique animal with multiple technical replicates), statistical significance was determined using a one-way ANOVA, where $*p<0.05$ was considered statistically significant. Scale bar: 50µM

Functionality of this model was confirmed by PCR analysis for excision of the Rb floxed allele and presence of the LXCXE binding domain mutation (Figure S4.1B).

Upon acute deletion of Rb in cortical neurons, we previously reported a disruption of quiescence as evidenced by the ectopic induction of cell-cycle related genes (Andrusiak et al., 2012). We hypothesized that if Rb maintains the long-term repression of these factors through interaction with LXCXE-dependent chromatin modifying enzymes, that a form of Rb defective in these interactions would be insufficient to maintain neuronal quiescence. We examined expression of the cell cycle related factor CycA2 by western blot at 10DIV (Figure 4.2A). There was a significant induction following complete Rb deletion ($Rb^{Flox/Flox}$); however, one $Rb^{\Delta L}$ allele ($Rb^{Flox/\Delta L}$) was sufficient to maintain CycA2 protein levels at control ($Rb^{Flox/+}$) levels (Figure 4.2A). Similarly, when we examined cortical neurons at 10DIV by immunofluorescence, we did not see any significant increase in Ki67+ cells in $Rb^{\Delta L}$ cells compared to control (Figure 4.2B).

We next examined the expression of cell cycle related genes *in vivo*. Due to the technical time-limitations on maintaining cultured cortical neurons, we hypothesized that lengthening our analysis may reveal gene expression defects in $Rb^{\Delta L}$ neurons. An inducible CamkCreERT2 model was used to delete the floxed Rb allele upon tamoxifen administration in adult post-mitotic cortical neurons (Figure 4.2C) (Andrusiak et al., 2012; Erdmann et al., 2007). Animals were injected with tamoxifen and examined four-weeks later, a time at which we previously showed massive neurodegeneration upon Rb deletion (Andrusiak et al., 2012). Animals deficient in Rb expression displayed ectopic expression of the cell cycle related factors Ki67 and CycE, whereas $Rb^{\Delta L}$ and control neurons did not show any significant changes (Figure 4.2C). This suggests that the Rb LXCXE-binding

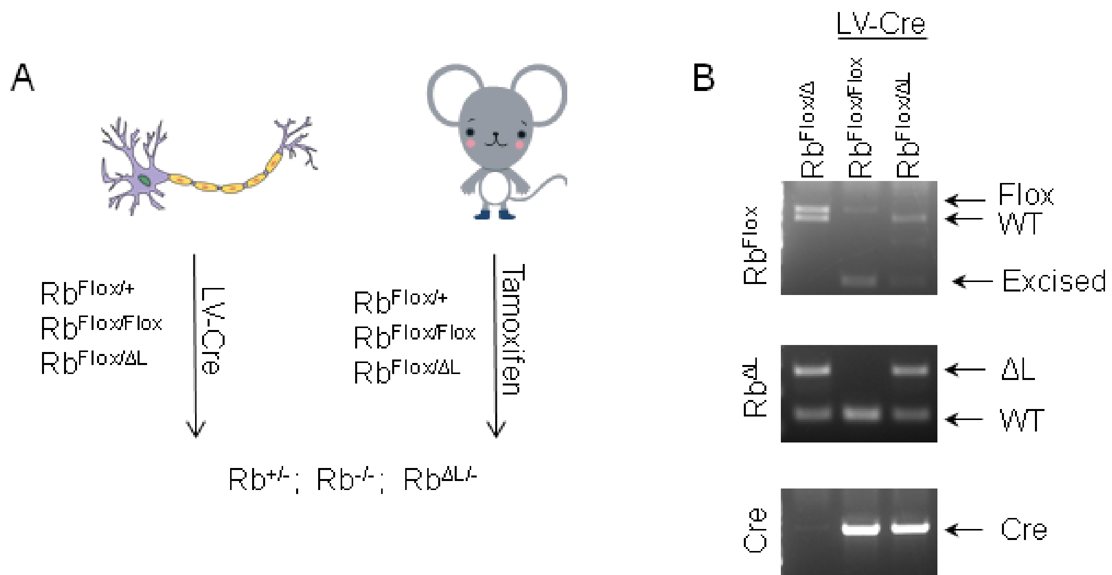

Figure S4.1. *Acute Rb LXCXE-binding domain deletion* <u>model</u> A) Representation of our *in vitro* and *in vivo* acute $Rb^{\Delta LXCXE}$ <u>model</u> B) PCR genotyping analysis of neurons of the indicated genotypes. Genotyping was performed for the presence of Cre, $Rb^{\Delta L}$ mutation and the floxed allele of Rb (intact and excised forms).

domain is dispensable for the long-term repression of cell cycle related factors. In addition, we employed a conditional model to address the ability of $Rb^{\Delta L}$ to establish neuronal quiescence by removing Rb expression in embryonic neural precursors (Figure S4.2). Consistent with previous results, the complete loss of Rb leads to ectopic proliferation in the developing dorsal cortex at E15.5 (Ferguson et al., 2002), as evidenced by Ki67 expression, whereas $Rb^{\Delta L}$ and controls did not display ectopic Ki67 (Figure S4.2).

The Rb LXCXE-binding domain is dispensable for neuronal survival

The most profound phenotype observed upon acute Rb deletion in cortical neurons was neurodegeneration. Cortical neurons present a unique contrast to other neuronal systems, as they are unable to tolerate loss of Rb (Ajioka et al., 2007). To examine whether survival may be impacted independently of quiescence in our LXCXE mutation model; we performed studies to assess neuronal survival. We examined neurons at 10DIV for induction of apoptosis by both microscopic assessment of condensed nuclei and western blot analysis for activated caspase-3 (AC-3) (Figure 4.3A-B). We did not observe any significant changes in survival by either of these two techniques in $Rb^{\Delta L}$ cells when compared to control (Figure 4.3A-B). To assess the potential for a more long-term contribution of $Rb^{\Delta L}$ on survival, we utilized our CamKCreERT2 model. Mice were examined for neuronal survival by quantifying the percentage of neurons (NeuN+) (Figure 4.3C). We observed massive neurodegeneration following Rb deletion, whereas no reduction in the number of neurons was observed in $Rb^{\Delta L}$ brains when compared to control (Figure 4.3C). Overall these results suggest that Rb interactions with the LXCXE-binding motif are dispensable for neuronal survival.

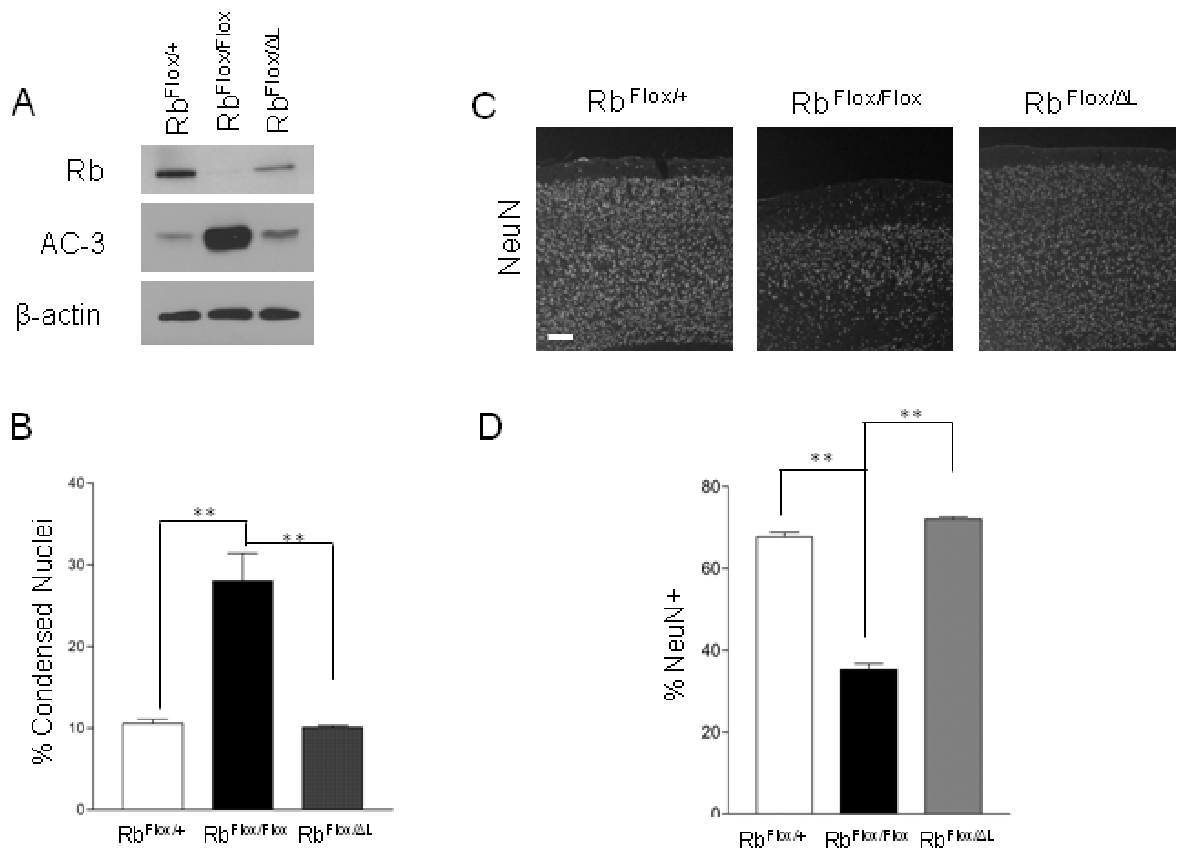

Figure 4.3. *Rb regulates neuronal survival in an LXCXE independent manner* A) Western blot analysis of total protein extracted from Control, Rb-deficient and $Rb^{\Delta L}$ cortical neurons at 10DIV B) Cortical neurons of the indicated genotypes were infected with lentiviral Cre and fixed at 10 DIV and condensed nuclei were examined by DAPI staining. Error bars represent SEM (n=3, where n represent an independent culture with multiple technical replicates), statistical significance was determined using a one-way ANOVA, where $*p<0.05$ was considered statistically significant. C) Animals of indicated genotypes were injected with tamoxifen (180 mg/kg/day i.p., 3 days) and euthanized 4weeks following the final injection. Representative pictures of NeuN immunofluorescence in the cortex of CamKCreERT2; $Rb^{Flox/Flox}$mice compared to CamKCreERT2; $Rb^{Flox/+}$ and CamKCreERT2; $Rb^{Flox/\Delta L}$mice. Scale bar: 100µm. D) NeuN+ cells were quantified in the cortex and percentages were obtained by normalizing to total DAPI cells for indicated genotypes. Error bars represent SEM (n=3, where n represents the cortex from a unique animal with multiple technical replicates), statistical significance was determined using a one-way ANOVA, where $*p<0.05$ was considered statistically significant.

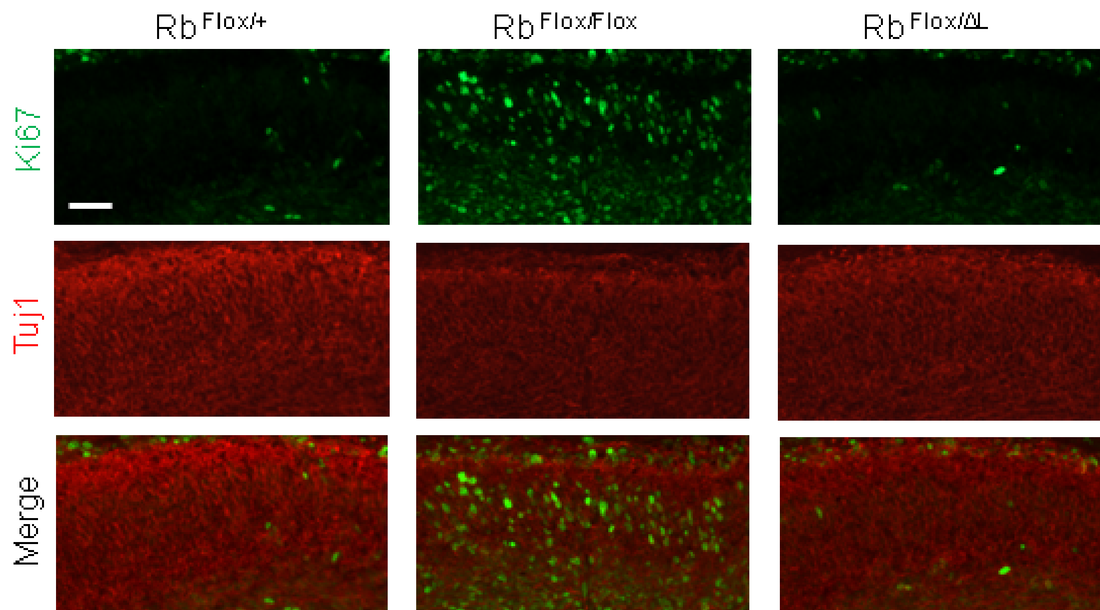

Figure S4.2. *Rb regulates the establishment of neuronal quiescence in an LXCXE independent manner*. Timed pregnant females were euthanized at E15.5. Representative pictures displaying ectopic proliferation (Ki67+) in immature neurons (Tuj1+) in the dorsal cortex of Foxg1-Cre; $Rb^{Flox/Flox}$mice compared to Foxg1-Cre; $Rb^{Flox/+}$ and Foxg1-Cre; $Rb^{Flox/\Delta L}$mice. Scale bar: 100µm

Induction of an active chromatin state occurs downstream of E2f-activation

No defects in the maintenance of quiescence or survival were found in cortical neurons in our acute $Rb^{\Delta L}$ mutant model. In relation to our initial observation, that in the absence of Rb chromatin is remodeled to an activate state, we asked whether this process was solely dependent on Rb or relied on E2f activation downstream of Rb loss. The role of the Rb/E2f pathway in chromatin remodeling is not limited to pocket protein mediated repression but has also been shown to include E2f-induced chromatin activation (Ait-Si-Ali et al., 2000; Taubert et al., 2004; Trouche et al., 1996). To test this, we utilized a dominant negative DP1 construct ($DP1_{\Delta 103\text{-}126}$) (Andrusiak et al., 2012; Park et al., 2000; Wu et al., 1996). As E2f-inhibiton was sufficient to rescue neuronal survival following Rb loss (Andrusiak et al., 2012), we now asked whether $DP1_{\Delta 103\text{-}126}$ was also sufficient to prevent chromatin remodeling. We hypothesized that if Rb/E2f mediated gene repression relies solely on an interaction between Rb and E2f that this construct would prevent any remodeling. If chromatin remodeling was dependent on Rb itself, the absence of these factors would result in changes to the chromatin landscape in the presence of $DP1_{\Delta 103\text{-}126}$. Western blot analysis revealed a restoration of PCNA and CycA2 protein to control levels (GFP) in $DP1_{\Delta 103\text{-}126}$ infected $Rb^{Flox/Flox}$ cortical neurons (Cre and $DP1_{\Delta 103\text{-}126}$) (Figure 4.4A). Chromatin immunoprecipitation was performed for Acetyl-H3 and H3K9me3 on $Rb^{Flox/Flox}$ cortical neurons infected with LV-GFP, LV-Cre or LV-Cre and $DP1_{\Delta 103\text{-}126}$. We examined the enrichment for these chromatin marks at the promoters of *PCNA* and *CycA2* (Figure 4.4B-C). We did not observe any significant differences in either mark at the *PCNA* or *CycA2* promoters in LV-GFP or LV-Cre/LV-$DP1_{\Delta 103\text{-}126}$ (Figure 4.4B-C). These results indicate that the change in chromatin activation state that is observed upon Rb removal is

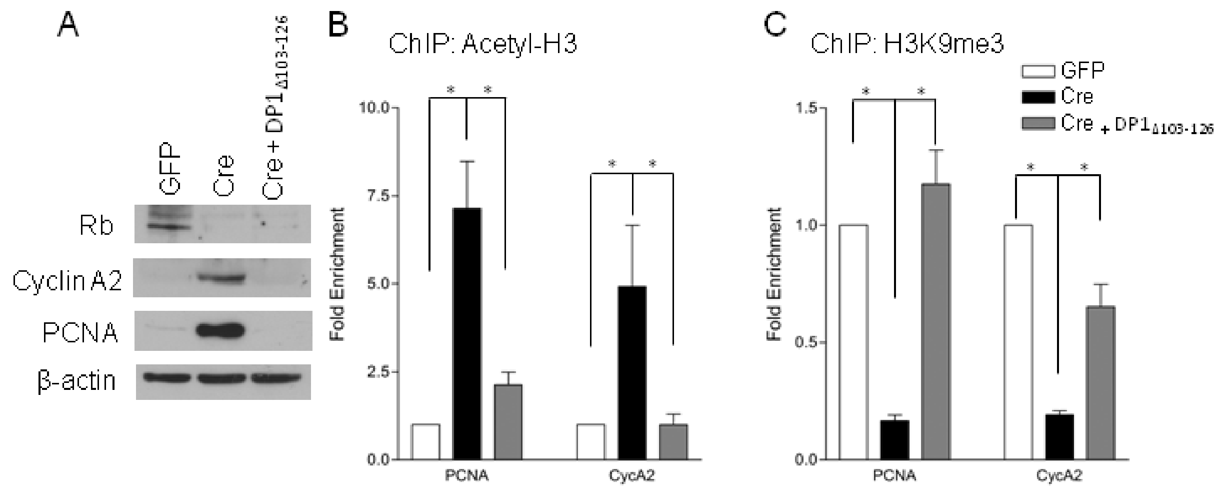

Figure 4.4. *Chromatin activation in the absence of Rb occurs at the level of E2f.* qRT-PCR analysis of the *PCNA* and *CycA2* promoters in GFP, Cre or Cre/ $DP1_{\Delta 103\text{-}126}$ infected cortical neurons ($Rb^{Flox/Flox}$) at 6DIV from chromatin immunoprecipitated DNA. A) Western blot analysis of total protein extracted from Control (GFP), Rb-deficient (Cre) and Rb-deficient/dominant negative DP1 (Cre and $DP1_{\Delta 103\text{-}126}$) cortical neurons at 6DIV B) Chromatin was immunoprecipitated with an antibody directed towards acetylated histone 3 (Acetyl-H3) C) Chromatin was immunoprecipitated using an antibody that recognizes tri-methylated lysine 9 on histone 3 (H3K9me3). Error bars represent SEM (n=3, where n represents a ChIP performed on neurons isolated from a unique embryo), statistical significance was determined using a one-way ANOVA, where $*p<0.05$ was considered statistically significant.

dependent on E2f-activation and not purely the removal of Rb. Our data therefore highlights the sufficiency of an interaction between Rb and E2f as a driving force in the maintenance of neuronal quiescence and survival.

Discussion

Our studies demonstrate a number of important conclusions regarding the role of Rb/E2f in the regulation of gene expression in post-mitotic cortical neurons. We have shown that acute loss of Rb results in remodeling of chromatin at E2f-responsive promoters to an active state. Surprisingly, we observe that long-term gene repression by Rb occurs independently of the ability to interact with LXCXE motifs that are found in many chromatin regulating enzymes. Using an acute model, we did not observe any changes, *in vitro* or *in vivo,* in the capacity of $Rb^{\Delta L}$ to mediate physiological changes or gene repression. Finally, we provide evidence that E2f-activation downstream of Rb deletion is essential in chromatin remodeling. In the absence of Rb, a dominant negative $DP1_{\Delta 103\text{-}126}$ mutant is able to maintain chromatin in an inactive state. These findings suggest that cell cycle gene repression by Rb/E2f in cortical neurons occurs at the level of E2f.

The initial finding that viral oncoproteins required the LXCXE motif in order to inactivate Rb suggested a critical role for this domain in Rb function. Accumulating evidence is now emerging that it is largely dispensable and only plays context specific roles in Rb function (Bourgo et al., 2011; Chan et al., 2001; Talluri et al., 2010). An elegant study in the liver, using acute transgenic and viral models, showed that the Rb LXCXE-binding domain is not required for basal transcription (Bourgo et al., 2011). In the liver, Rb-LXCXE interactions were only required after treatment with a genotoxic agent, where it was shown

that these animals upregulate cell-cycle related targets and as a result, initiate hepatocellular carcinomas (Bourgo et al., 2011). This study complemented initial work revealing that $Rb^{\Delta L}$ mutation, in a p53-null background, resulted in more aggressive tumor formation due to genomic instability created by defects in chromosome condensation (Coschi et al., 2010). Rb-LXCXE interactions have also been shown to be essential for stress-responsive G_1 arrest or senescence (Narita et al., 2003; Talluri et al., 2010). $Rb^{\Delta L}$ mutants showed specific defects in H3K9me3 recruitment at heterochromatin, a hallmark of senescence (Isaac et al., 2006; Narita et al., 2003; Talluri et al., 2010). Furthermore, studies have implicated Rb in the recruitment of H3K4 demethylases, Jarid1a and Jarid1b, specifically during senescence (Chicas et al., 2012). These studies suggest a unique mechanistic role for Rb in stress-associated cell cycle arrest compared to cell-cycle exit during terminal differentiation (Chicas et al., 2012; Talluri et al., 2010). Both processes are Rb-dependent; however, senescence uniquely requires LXCXE-dependent factors (Chicas et al., 2012; Talluri et al., 2010). Though senescence and cortical neuronal differentiation are thought to be permanent cell cycle withdrawal events, our data addresses the differential mechanism employed by Rb in the latter. Studies in retinal neurons may not represent an ideal neuronal population to study terminal quiescence, as these neurons have been shown to de-differentiate and proliferate (Ajioka et al., 2007; Talluri et al., 2010). Furthermore, our acute model adds a level of specificity to help prevent compensation from other pocket protein family members.

Our data supports the notion that Rb-mediated gene repression occurs independently of LXCXE interactions, though it does not exclude the possibility that factors interacting through other Rb-domains influence gene repression. Our dominant negative $DP1_{\Delta 103\text{-}126}$ model strengthens the conclusion that chromatin remodeling does occur downstream of Rb

regardless of LXCXE or non-LXCXE factors. In this experiment we completely removed the Rb protein thus all other accessory factors. The addition of $DP1_{\Delta 103\text{-}126}$ was sufficient to block chromatin activation, suggesting that Rb-interacting factors are either 1) not present in cortical neurons or 2) present but dispensable in this context. Studies have implicated the switch between repressive (histone deacetylase containing) and activator (histone acetyltransferase containing) complexes during the switch to E2f-activation (Frolov and Dyson, 2004). Little is known about the specific role for activator E2fs (E2f1-3) in recruitment of these complexes. Studies in *Drosophila* have shown that E2f-activation is a two-step process, with the initial disruption of Rb-dependent repressor complexes and then recruitment of E2f-activation complexes (Frolov et al., 2003). Evidence has been presented to suggest the relationship between E2f-activation and histone acetyltransferase recruitment (Ait-Si-Ali et al., 2000; Taubert et al., 2004; Trouche et al., 1996; Trouche and Kouzarides, 1996); however, further studies probing these interactions in an *in vivo* physiological context would be extremely informative.

In conclusion, our results reveal a specific role for Rb-E2f interactions in the maintenance of gene repression in cortical neurons. We found that Rb-mediated gene repression occurs independently of its LXCXE-binding domain. We did not observe any physiological or gene expression changes when neurons had to acutely rely on $Rb^{\Delta L}$. Our use of a dominant negative $DP1_{\Delta 103\text{-}126}$ construct revealed that chromatin remodeling after Rb deletion occurs at the level of E2f. These findings reveal the need for constitutive inhibition of E2f in order to maintain gene repression and neuronal survival. It also highlights the differential role Rb plays in terminally differentiated quiescent neurons when compared to previous studies investigating stress-induced cell cycle arrest (Andrusiak et al.,

2012; Bourgo et al., 2011; Isaac et al., 2006; Talluri et al., 2010). Most importantly our results provide evidence that in terminally quiescent neurons, Rb/E2f function is more dependent on E2f activity than previously thought.

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Materials and Methods

Animals

All experiments were approved by the University of Ottawa's Animal Care Ethics Committee adhering to the Guidelines of the Canadian Council on Animal Care. Rb^{Flox} (Marino et al., 2000) and Foxg1-Cre(Hebert and McConnell, 2000) were maintained on an FVBN background while $Rb^{\Delta LXCXE}$ (Isaac et al., 2006) and CamKCreERT2 (EMMA ID: 02125)(Erdmann et al., 2007) mice were maintained on a C57Bl/6 background. Animals were genotyped according to standard protocols with previously published primers. For *in*

vitro experiments $Rb^{Flox/Flox}$ animals were crossed, or $Rb^{Flox/\Delta LXCXE}$ to $Rb^{Flox/+}$. All Cre-expressing animals used were heterozygotes for Cre expression. CamKCreERT2;$Rb^{Flox/+}$ mice were crossed with $Rb^{Flox/\Delta LXCXE}$ mice to generate experimental animals, which at 5-6 weeks of age, were given tamoxifen (Sigma) (180 mg/kg/day i.p., 3 days) and euthanized four weeks after the final injection.

Primary Cortical Neurons

Embryonic cortical neurons were isolated by standard procedures.(Fortin et al., 2001) Neurons were infected at the time of plating with a pWPXLD lentiviral vector expressing control GFP, GFP-tagged Cre recombinase or dominant negative $DP1_{\Delta 103\text{-}126}$ at a multiplicity of infection (MOI) of 2. For immunofluorescence, cells were grown on coverslips for 10DIV then fixed with 4% paraformaldehyde (PFA) and stained for Ki67 (Cell Marque, Cat No. 275-16), DAPI and visualized with AlexaFluor 488 secondary antibody (Invitrogen, Cat No. A11055). Statistical differences were determined using a one-way ANOVA, where $p<0.05$ was considered statistically significant.

Chromatin Immunoprecipitation

Cortical neurons were cross-linked with 1% formaldehyde for 10min at room temperature. Neurons were lysed (50mM Tris-HCl, 1% SDS, 10mM EDTA, Protease Inhibitor Cocktail (Sigma)), sonicated for 30min (30s (on), 45s (off)) using a BioRuptor® (Diagenode), and centrifuged at 14,000 x *g* to remove cellular debris. Each immunoprecipitation was performed using 2μg of antibody directed to acetylated histone 3 (Millipore, Cat No. 06-599), tri-methylated lysine 9 on histone 3 (Abcam, Cat No. ab8898) or normal rabbit immunoglobulin G (Millipore, Cat No. 12-370). Immunocomplexes were captured using

Dynabeads® Protein A (Invitrogen) and washed extensively. Cross-links were reversed overnight, followed by treatment with RNase A at 37°C for 1 h and proteinase K at 65°C for 30 min. Purified DNA was analyzed by real-time PCR using PerfeCTa SYBR Green FastMix Reaction Mix (Quanta Biosciences) and a Rotor-Gene RG-3000 (Corbett Research). Primer sequences are available upon request and were designed to encompass E2f binding regions in the *PCNA* and *CycA2* promoters. Expression values were obtained from three immunoprecipitations from three independent cultures and significance was determined using a one-way ANOVA, where $p<0.05$ was considered statistically significant.

Western Blots

Protein was isolated from cultured cortical neurons and western blot analyses performed as previously described(Ferguson et al., 2002) with antibodies directed towards cleaved caspase-3 (Cell Signaling, Cat No. 9664S), Rb (Pharmingen, Cat No. 554136), Cyclin A2 (Abcam, Cat No. ab7965), PCNA (Millpore, Cat No. MAB424) and β-actin (Sigma, Cat No. A-5316).

Tissue processing, immunohistochemistry and cell quantification

Brains were perfused and fixed as previously described.(Fortin et al., 2001),(Ferguson et al., 2002) Sections were collected as 14-µm coronal cryosections on slides. For immunohistochemistry, sections underwent antigen retrieval in Target Retrieval Solution (Dako) and incubated overnight at 4°C with the following primary antibodies: NeuN (Encor Biotechnology, Cat No. MCA-1B7), Tuj1 (Covance, Cat No. MMS-435P), Ki67 (Cell Marque, Cat No. 275-16) and Cyclin E (Santa Cruz, Cat No. sc-481). Sections were incubated in blocking solution containing donkey anti-rabbit AlexaFluor488 (Invitrogen,

Cat No. A11055) or donkey anti-mouse Cy3 (Jackson Immunoresearch, Cat No. 715-165-150) and DAPI. All images were acquired using a Zeiss Axioobserver D1. For cell quantification, a minimum of 3 sections containing the frontal cortex were analysed per brain and the percentage of positive (NeuN, Ki67 or Cyclin E) cells among the total DAPI+ cells were quantified. Statistical differences were determined using a one-way ANOVA, where $p<0.05$ was considered statistically significant.

REFERENCES

As per the guidelines "Writing a M.Sc. or Ph.D thesis" from the Department of Cellular and Molecular Medicine at the University of Ottawa, Sub-section "Format of a Collection of Articles", references of all sections are listed in an amalgamated list at the end of the thesis.

http://www.intermed.med.uottawa.ca/cellmed/eng/writing.html

CHAPTER 5- DISCUSSION

5.0 Closing One Door, Opening Another: Cell Cycle Re-Entry From a Differentiated State

The data presented in this thesis support a number of important conclusions regarding the cell-type specific role for Rb in the central nervous system. First, using the defects in tangential migration as a model of non-cell cycle dysfunction upon Rb deletion, we validated Rb/E2f regulation of neogenin and implicated it in this process. Secondly, we reveal that in quiescent cortical neurons, Rb plays a more one-dimensional role by primarily repressing cell cycle associated genes. This is a stark contrast to the minimal cell cycle de-regulation observed in ventral forebrain progenitors in the absence of Rb (McClellan et al., 2007). Finally, we observed an induction of active-chromatin at E2f-responsive promoters in the absence of Rb. Using an acute LXCXE-binding deficient mutant paradigm; we report that the LXCXE binding motif is dispensable in establishment and maintenance of cortical neuron quiescence and survival. We describe a dependence on the activation state of E2f as being pivotal in the change in chromatin-state and not simply the loss of Rb or associated co-factors. **Taken as a whole this thesis serves to support the hypothesis that Rb plays diverse roles in different cell-types by regulation of unique gene targets and regulatory mechanisms.** The significance of these results are discussed in the conceptual framework of how differential facets of Rb and E2f function can act individually, or perhaps in concert, to regulate complex pathological events such as neurodegeneration and tumourigenesis.

5.1 Cell Cycle Re-Entry as a Prelude to Neurodegeneration

The re-expression of cell cycle proteins in neurons, or cell cycle re-entry, has been observed in Alzheimer's disease (AD) and other neurodegenerative conditions (reviewed in Herrup and Yang, 2007). The paradoxical ease of cell cycle reactivation in a seemingly

terminally quiescent neuronal population suggests the need for constant repressive action on cell cycle components in order for their expression to remain silenced. Recent experimental models of neurodegeneration have implicated cell cycle re-entry as a *bona fide* upstream event in disease progression (Andorfer et al., 2005; Lee et al., 2009; Varvel et al., 2009), though the intersection between pathogenic neurodegenerative components such as amyloid beta and reactivation of the cell cycle machinery has yet to be fully determined. It is still a matter of debate if ectopic expression of cell cycle proteins is a cause or effect of neuronal loss (Lopes et al., 2009; Wang et al., 2007). Determining the molecular machinery by which quiescence is maintained in mature neurons will aid in the understanding of the events initiating neuronal loss. Our data suggest that Rb deletion in post-mitotic neurons models pure cell cycle re-entry, suggesting that this process itself is capable of inducing neuronal apoptosis.

Many deletion models for Rb are confounded by compensation by the other pocket proteins; it has been shown that in the absence of all three pocket proteins, neural precursor cells are able to properly exit the cell cycle, suggesting even non-pocket protein compensation (Wirt et al., 2010). Acute paradigms are therefore extremely informative in the dissection of specific functions for Rb family proteins. In the retina, fully differentiated horizontal neurons were able to re-enter and divide while maintaining their neuronal characteristics in the absence of pocket proteins suggesting, within the neuronal lineage, a differential dependence on Rb family members and different responses to ectopic proliferation (Ajioka et al., 2007). In differentiated myotubes, Rb down-regulation was sufficient to up-regulate cell-cycle proteins and reduce expression of myogenic factors (Blais et al., 2007). The down-regulation of myogenic factors triggered a ‘de-differentiation’

of these mature myotubes to a more immature proliferative myoblast-state (Blais et al., 2007). In our system, no changes were observed in proneural or progenitor factors, suggesting a more defined role for the retinoblastoma protein in cortical neurons. Our acute paradigm highlighted the specific role for the retinoblastoma protein in the maintenance of neuronal quiescence by the repression of core components of the cell cycle machinery. This is a stark contrast to other tissue systems in which Rb plays a multi-facted role in the repression of proliferative, apoptotic and fate specification genes (reviewed in Viatour and Sage, 2011). Additionally, we were able to translate the phenotypic consequences of pure cell cycle re-entry, induced by Rb loss into neurodegeneration, both *in vitro* and *in vivo*.

In the context of AD, previous studies demonstrated that beta-amyloid (Aβ) oligomers could induce cell cycle re-entry and cell death in cortical neurons *in vitro,* as well as promote Rb phosphorylation (Biswas et al., 2007). Higher levels of phosphorylated Rb have been detected in the brain of AD patients (Hoozemans et al., 2004; Stone et al., 2011; Thakur et al., 2008). These aforementioned studies were largely correlative and the direct relationship between Rb and neurodegeneration *in vivo* remained unclear. Transgenic mice expressing the SV40 large T antigen under the CaM kinase IIα promoter, in turn inactivating the entire pocket protein family (Rb, p107 and p130) and p53 *in vivo*, displayed neuronal cell cycle re-entry and degeneration (Park et al., 2007). We expand upon these findings by displaying that the loss of Rb is sufficient to trigger activation of the cell cycle machinery and subsequently cell death. This supports the hypothesis that phosphorylation of Rb is a causative factor in neurodegeneration, and not just an indirect consequence of neuronal loss.

Neurodegenerative studies have focused on re-entry in excitatory populations, with little emphasis being placed on inhibitory interneurons like those implicated in

retinoblastoma. It would be informative to analyse the effects of Rb-deletion on cortical interneuron homeostasis. Retinal interneurons appear to tolerate ectopic proliferation (Ajioka et al., 2007), for this reason examining Rb loss in cortical interneurons may be informative. Discerning whether intrinsic cues such as p53-activation dictate the response to proliferative events, or simply the structural ability of a polarized cell type to tolerate division elicits a differential cell-type specific apoptotic response. Data supplementary to this thesis also suggests that Rb regulates subtype specific functions in developing interneurons (Ghanem et al., 2012). Rb/E2f regulated gene targets may differ within the excitatory and inhibitory neuronal populations. It would be useful in the context of both neurodegeneration and cancer, to examine Rb function thoroughly in cortical interneurons.

5.2 Roles for Rb in Cancer: Clarifying Old and Identifying New

The classical role of the retinoblastoma regulation of G_1/S progression in cancer progression is well established (reviewed in Burkhart and Sage, 2008). The paradoxical absence of proliferative disruption within rapidly-amplifying progenitor populations in models of retinoblastoma and other Rb-associated tumours, suggests a function beyond expansion of proliferating cell types in the absence of Rb. With the hallmarks of cancer expanding from the initial six pivotal aspects of tumourigenesis (Hanahan and Weinberg, 2011), we now have evidence for the participation of Rb in nearly every facet of cancer progression. This discussion will focus on novel aspects of Rb in tumour progression and the notion of hybrid cellular states as a driving force in Rb-mediated disease.

Our finding that Rb/E2f directly regulates the expression of neogenin, a receptor implicated in axon guidance has two important implications. Our finding that Rb deletion

disrupts tangential migration during neurogenesis was important although, the broader implications in cancer biology may be significantly more impactful. It is now emerging that axon guidance factors play an important role in the pathogenesis of many cancers outside of the CNS (reviewed in Mehlen et al., 2011). This provides correlative evidence that, not only does Rb regulate a factor involved in developmental migration, but factors such as neogenin may also influence tumour cell invasion and migration. A comprehensive genomic approach in pancreatic cancer revealed that, along with common known disease associated mutations (Ras, p53, p21), a significant portion of patients also had mutations within axon guidance pathways (Slit/Robo) (Biankin et al., 2012). Specific evidence for neogenin and its related receptor DCC has also been presented during CNS and non-CNS tumorigenesis. Neogenin has been implicated in regulating various facets of angiogenesis, a critical cancer hallmark process (Eveno et al., 2011; Lee et al., 2005; Lejmi et al., 2008). DCC has a more broad role, ranging from classical migration associated processes to cellular differentiation (reviewed in Duman-Scheel, 2009). Our data suggests that Rb/E2f regulation of neogenin may play a role in the progression of certain tumour types. In a *murine* inducible transgenic glioma model, it was shown that deletion of Rb, in addition to other tumour suppressor pathways (p53, PTEN), induced formation of a unique invasive primitive neuroectodermal tumour (Jacques et al.). These tumours are derived from neural precursors, the population in which we showed Rb/E2f to regulate neogenin expression. It is suggested that certain tumours, such as retinoblastoma, emerge from more differentiated cell types. In more differentiated cortical types, we did not observe any induction of neogenin or other migratory factors. It is essential to identify the initiating population in order to discern what cell-type specific effects Rb dysfunction may elicit. In the context of forebrain cellular

populations, *RB1* mutation could have migratory implications depending on the timing of Rb loss. A resounding question remains in finding out what triggers differential expression by Rb/E2f factors such as neogenin. Examining the epigenetic state or occupancy of adjacent promoter regions may be informative in discovering why Rb/E2f exerts tissue specific effects.

Another critical Rb-associated process and hallmark of cancer is the ability to evade cell death. This thesis serves to answer several questions regarding the role of Rb in cell death yet also presents many more. Similar to the reciprocal regulation of neogenin expression in progenitor and neuronal populations, it seems as though Rb/E2f effects cell viability in a cell-type specific fashion. Previous data has suggested that Rb or pocket protein inactivation in neural precursor cells resulted in a cell death response (Marino et al., 2003; Slack et al., 1998; Slack et al., 1995). Both initial reports of Rb-deletion in the developing forebrain challenged the apoptotic phenotypes associated with Rb loss (Ferguson et al., 2002; MacPherson et al., 2003). Conversely, retinal progenitors display increased apoptosis after Rb or pocket protein deletion (Ajioka et al., 2007; Chen et al., 2007; Robanus-Maandag et al., 1998; Xu et al., 2009). We report a differential cell death response to Rb deletion in cortical precursors and neurons. In precursors, no overt changes in cell death are observed. In contrast, cortical neurons initiate a latent apoptotic cascade, likely due to DNA damage pathway activation. We hypothesize that DNA damage induction in cortical neurons results from an inability to process or sustain proper cell cycle activity. In a cell that is proliferatation-incompetent many factors may lead to dysfunction after cell cycle re-entry. Reports have implicated events such as DNA re-replication (Burhans et al., 2002) and nucleotide imbalances (Bester et al., 2011; Harwood et al., 1996), in initiating apoptosis

downstream of cell cycle activation. Both of these events intersect with the notion that genomic instability is an essential factor in many Rb-regulated apoptotic events and perhaps tumour progression. Importantly, in our model of Rb-deletion and E2f hyper-activation, we do not observe a transcriptional apoptotic response but latent cell death downstream of DNA damage.

Genomic or chromosomal instability has been observed in cancerous and precancerous cell-types, though the specific contributions that it plays during tumourigenesis are still unknown (reviewed in Schvartzman et al., 2010). Events leading to genomic instability are becoming more well-defined, with Rb-loss and dysfunction being a key initiating event (reviewed in Coschi and Dick, 2012). Our model of double strand breaks in a post-mitotic state suggests that increased E2f activity is causal in inducing DNA damage. Though we were not able to empirically test this hypothesis, it is likely that replication or mitotic defects downstream of increased E2f-activity leads to DNA damage and apoptosis in cortical neurons. In models of Rb-mediated genomic instability, more subtle defects need to occur in order to evade an apoptotic response. Ascertaining the role of Rb/E2f and genomic instability in cancer progression may be more directly studied in models of upstream pathway dysfunction. Hyperactive CDK4/Cyclin D or reduced p21 expression would model Rb-inactivation while still leaving a functioning protein to act during mitotic progression. There is evidence to suggest that an Rb defective in binding E2f is sufficient to prevent prostate cancer tumourigenesis, though the aspect of genomic instability was not examined (Sun et al., 2011). This study would suggest that a non-E2f function, possibly through regulation of mitotic fidelity, is a contributing factor to Rb-deficiency induced tumour formation.

The absence of mitotic progression in cortical neurons may explain the ability of LXCXE-deficient Rb to compensate during quiescence. All evidence from *in vivo* models suggests that LXCXE-interactions are important during mitotic progression and after genotoxic stress. The addition of genotoxic stress may act in concert with LXCXE-deficiency in these models to create additional genomic instability. The use of our acute model of Rb LXCXE binding deficiency in neural precursors would be informative to assess the potential contribution of 1) Rb regulation of mitotic fidelity in the CNS 2) The impact of potential mitotic defects and genomic instability created on neuronal function 3) Intersection with other known oncogenes or tumour suppressors in CNS cancer progression. LXCXE deficiency may provide the unexpected tool of examining the role of Rb in genomic instability, as opposed to the previously hypothesized function in gene repression.

One of the greatest resounding questions in the field of retinoblastoma and many cancer-types is: what cell-type is driving the growth of the tumour? In the context of retinoblastoma, it is easy to assume that aberrant proliferation of retinal precursors is the causative factor. This population is rapidly dividing and Rb loss may serve to eliminate the G_1/S transition increasing division rates to a pathological level. This aforementioned hypothesis is likely not the case in retinoblastoma. Few reports have observed increases in retinal progenitor proliferation in transgenic retinoblastoma models (McEvoy et al., 2011; Vooijs et al., 2002; Xu et al., 2009). The common theme in these models is the experience of ectopic cell division in differentiated post-mitotic retinal neurons (McEvoy et al., 2011; Vooijs et al., 2002; Xu et al., 2009). The questions that remain to be answered are 1) Are these retinal neurons failing to properly exit the cell cycle and undergoing extra cell divisions? 2) Are retinal neurons exiting the cell cycle then re-entering from a differentiated

state and dividing? There are several lines of evidence, including that from this thesis that support both models.

The concept of cellular de-differentiation is not new in the field of tumourigenesis; however, only recently has evidence been put forth to suggest this may occur in retinoblastoma. This stemmed from an initial finding that early-stage retinoblastomas displayed a high-degree of differentiation (Johnson et al., 2007). This is uncommon in most neurological tumours, as they are proposed to have a more immature proliferative phenotype. In these early-stage tumours, retinal neurons expressed proper sub-type markers and extended correct processes (Johnson et al., 2007). As the disease progressed, these mature retinal neurons disappeared and the tumour began to appear de-differentiated (Johnson et al., 2007). A subsequent study implicated fully mature horizontal cell de-differentiation as the initiating population in the disease (Ajioka et al., 2007). Molecularly, these de-differentiated retinal neurons displayed a mix of neuronal and immature factors, resulting in a hybrid retinoblastoma cell-type (McEvoy et al., 2011). Though technically challenging, it was not shown that these mature horizontal neurons could develop tumours upon xenograting into immunocompromised animals. This lends credence to the notion that de-differentiation is the driving force in retinoblastoma; however, work from this thesis and other studies question these conclusions.

In our forebrain model, we indeed observe similar events in the absence of Rb as the retina. There is no significant change in progenitor cell proliferation, and immature neuronal sub-types exhibit ectopic expression of proliferative markers. Previous models of Rb-deletion could not assess the postnatal contribution of these cells, as the animals did not survive beyond birth (Ferguson et al., 2002). In our acute Rb-deletion in post-mitotic

neurons, we are able to show that this quiescent population is unable to tolerate cell cycle re-entry and undergo apoptosis. Though these are a different neuronal subtype, it suggests that from a mature state cell cycle re-entry is not tolerated. This reinforces the notion that cell cycle exit delay is likely the causative factor in retinoblastoma progression. In this scenario, immature neurons require a compensatory factor (p107, p130, E2f7/8) to allow for proper cell cycle exit. Once the appropriate E2f-targets are repressed then the cell undergoes cell cycle exit, after potentially several rounds of ectopic division. This hypothesis is supported from several models of retinoblastoma.

An interesting observation during retinal development in mice came with the finding that Rb expression peeks in maturing retinal cone progenitor cells (Xu et al., 2009). This complemented one of the initial transgenic retinoblastoma models, which suggested that pocket protein loss resulted in an exit delay that temporarily by-passed terminal differentiation (Chen et al., 2004). These tumours expressed markers of the inner nuclear layer, composed of interneurons, not the outer layer rod and cone composition (Chen et al., 2004). This would suggest that Rb is important in the timing of cell cycle exit in cone precursors. These tumours did not contain any mature cone cells, suggesting that these cells fail to properly differentiate (Xu et al., 2009). Subsequent xenografting of these cells was able to produce additional tumours in immunocompromised mice. This does not dismiss the notion that de-differentiation may be a factor; however, if it were causative, markers of mature cone cells should be observed, albeit at lower levels. A previous study also implicated progenitor cell exit delay, but observed broad delay across all populations. This study suggested that, in contrast to the model of de-differentiated proposed by the Dyer group, horizontal cells were present under these tumour conditions (Xu et al., 2009).

Though the aforementioned studies were at odds with the de-differentiation model, they again differed in their cell-type of origin, but supported data from this thesis and the exit-model of retinoblastoma.

Retinoblastomas consist of a mosaic of cell-types that comprise the tumour and tumour environment. Recent evidence has shown, using single-cell gene expression analysis, that cells derived from retinoblastomas exist in a hybrid-state (McEvoy et al., 2011). These cellular hybrids expressed factors associated with retinal progenitors and fully-differentiated retinal neurons (McEvoy et al., 2011). The notion of cellular hybrid, coupled with the overwhelming evidence to suggest cellular specific of Rb and E2f, lends itself to the question: Do Rb and E2f exist in a hybrid state during retinoblastoma development? One could envision a hybrid Rb/E2f state to select for traits that may predominant in one cell type that promote tumourigenesis, while those traits in different types that may be tumour suppressive to be selected against. The control of apoptotic genes would no longer be under E2f control, as observed in our cortical neurons though, pro-oncogenic genes such as neogenin or previously characterized factors such as Ezh2, Myb and FoxM1 (Bohrer et al., 2010; Catchpole et al., 2002; Wierstra and Alves, 2006) would be de-repressed following Rb loss. The culmination of a hybrid Rb/E2f state may be the perfect storm for tumour development and progression.

5.3 Summary

These studies have provided evidence that Rb plays a cell-type specific role. We provide data that the Rb/E2f pathway regulates diverse processes in neural precursor cells which include governing tangential migration by direct regulation of neogenin expression.

Once cells enter a post-mitotic state, Rb plays a more one-dimensional role in the constitutive repression of cell cycle genes to maintain neuronal quiescence and survival. Finally, we determine that regulation of gene repression and chromatin activation in the absence of Rb occurs independently of its LXCXE-binding domain. The activation state of chromatin at E2f-responsive promoters occurs downstream of E2f-activation. These results support further examination into cell-type specific functions in Rb/E2f function, most importantly their role in other neuronal populations. Determining the factors which govern tissue-specific Rb/E2f function is pivotal. The emerging data supporting heterogeneity of a pathway initially thought to regulate the progression from G_1 to S phase of the cell cycle is providing increased evidence to explain the pattern of tumourigenesis displayed in *RB1* and Rb/E2f pathway mutants.

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APPENDIX A- UNPUBLISHED SUPPLEMENTAL DATA

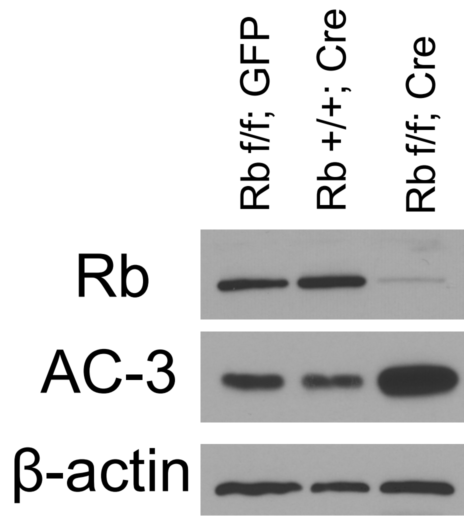

Figure A1. Infection of lentiviral cre does not result in apoptosis. Cortical neurons of the indicated genotypes were infected with GFP or Cre expressing lentivirus at an MOI of 2. Neurons were analyzed by western blot for expression of AC-3.

APPENDIX B- CURRICULUM VITAE

MATTHEW ANDRUSIAK

EDUCATION

University of Ottawa (Ottawa,ON)

Ph.D in Neuroscience **2008-**

Thesis: Differential roles for the retinoblastoma protein in proliferating and post-mitotic neural populations

Expected Graduation: May 2013

University of Ottawa (Ottawa, ON)

B.Sc Honours Co-op (Magna Cum Laude) **2003- 2008**

Area of concentration: Biochemistry

Thesis: The role of EphB4 in prostate cancer migration and invasion

AWARDS

Ontario Graduate Scholarship 2011 – 2013

CIHR Doctoral Poster Competition: Honorable Mention 2011

University of Ottawa Brain Health Research Day Poster Award 2011

Fisher Scientific Award of Excellence in Graduate Studies 2010

International Society for Developmental Neuroscience Bursary 2010

Heart and Stroke Graduate Studentship 2009 – 2011

Ontario Graduate Scholarship in Science and Technology 2008 – 2009

University of Ottawa Graduate Excellence Scholarship 2008 – Present

NSERC Summer Research Award 2008

Undergraduate Research Excellence Award 2008

Dean's List 2003 – 2008

University of Ottawa Undergraduate Admission Scholarship 2003 – 2008

RESEAECH EXPERIENCE

PhD Thesis Research, University of Ottawa 2008 -

Differential roles of the retinoblastoma protein in proliferating and post-mitotic neural populations

Honors Thesis Research, Ottawa Hospital Research Institute 2007 - 2008

The role of EphB4 in prostate cancer migration and invasion

Co-op Program Research, Ottawa Hospital Research Institute 2006 - 2007

Site-specific focal adhesion kinase phosphorylation during angiogenesis

PUBLICATIONS

Andrusiak MG, Vandenbosch R, Park DS, Dick FA, Slack RS. LXCXE-independent chromatin remodeling by Rb/E2f mediates neuronal quiescence. *Cell Cycle*. 2013 May 1;12(9) *In press*

Julian LM, Vandenbosch R, Pakenham CA, **Andrusiak MG** Nguyen A,McClellan KA, Svoboda DS, Park DS, Leone G, Blais A, Slack RS. Opposing Regulation of Sox2 by Cell-Cycle Effectors E2f3a and E2f3b in Neural Stem Cells.. Cell Stem Cell. 2013 Mar 13. doi:pii: S1934-5909(13)00056-8. 10.1016/j.stem.2013.02.001. [Epub ahead of print]

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McClellan KA, Vanderluit JL, Julian LM, **Andrusiak MG**, Dugal-Tessier D, Park DS, Slack RS. p107/E2F pathway regulates FGF2 responsiveness in neural precursor cells. *Mol Cell Biol*. 2009 Sep;29(17):4701-13. Epub 2009 Jun 29

ABSTRACTS (FIRST AUTHOR ONLY)

Andrusiak MG, Ghanem N, Julian LM, McClellan KA, deRepentigny Y, Kothary R, Park DS, Ekker M and Slack RS. The RB/E2F pathway modulates neurogenesis through regulation of the Dlx transcription factor. Second Biennial Retinoblastoma Meeting, Toronto, ON. November 2011

Andrusiak MG, Vandenbosch R, Ghanem N, McClellan KA, Park DS, Slack RS. The role of the Rb/E2F pathway in post-mitotic and proliferating neural populations. Brain Health Research Day, Ottawa, ON. June 2011

Andrusiak MG, Vandenbosch R, Ghanem N, McClellan KA, Park DS, Slack RS. The role of the Rb/E2F pathway in post-mitotic and proliferating neural populations. Canadian Student Health Research Forum, Winnipeg, MB. June 2011

Andrusiak MG, Ghanem N, Julian LM, McClellan KA, deRepentigny Y, Kothary R, Park DS, Ekker M and Slack RS. The RB/E2F pathway modulates neurogenesis through

regulation of the Dlx transcription factor. Keystone Symposia: Adult Neurogenesis, Taos, NM, USA. January 2011

Andrusiak MG, McClellan KA, Dugal-Tessier D, Kennedy TE, Park DS, Slack RS. Rb/E2F regulates neogenin expression during neuronal migration. International Society for Developmental Neuroscience, Estoril, Portugal. July 2010

Andrusiak MG, McClellan KA, Dugal-Tessier D, Kennedy TE, Park DS, Slack RS. Rb/E2F regulates neogenin expression during neuronal migration. First Biennial Retinoblastoma Meeting, Toronto, ON. November 2009

Andrusiak MG, McClellan KA, Dugal-Tessier D, Kennedy TE, Park DS, Slack RS. Direct regulation of neogenin by the pRb/E2F pathway plays a role in neuronal migration in the ventral telencephalon. Cold Spring Harbour/Wellcome Trust Mouse Genetics Meeting, Hinxton,UK. September 2009

Andrusiak MG, McClellan KA, Dugal-Tessier D, Kennedy TE, Park DS, Slack RS. Evidence for a novel netrin-neogenin interaction in pRb mediated interneuron migration. Great Lakes Mammalian Development Meeting, Toronto,ON. March 2009

Andrusiak MG, Addison CA. The Role of EphB4 in Prostate Cancer Migration. University of Ottawa Honour's Research Symposium. Ottawa, ON April 2008

PROFESSIONAL ACTIVITIES

Society for Neuroscience
Member — 2012- Present

Faculty of Medicine Seminar Series
Committee Member, Organization of cross-departmental distinguished seminar series — 2011 - Present

Let's Talk Science
Volunteer, Organize and participate in laboratory tours/seminars for High School students — 2010 - Present

Canadian Stroke Network Trainee Association
Member — 2010 - Present

Neuroscience/Cellular and Molecular Medicine Student Council
Member, Participate in the organization of departmental academic and social events — 2009 - Present

Ottawa Technology Venture Challenge
Participant, Draw plans for the creation and implementation of novel scientific technology — 2007 – 2008

APPENDIX B – PERMISSION TO REPRINT PUBLISHED MANUSCRIPTS

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Title: Rb/E2F Regulates Expression of Neogenin during Neuronal Migration

Author: Matthew G. Andrusiak, Kelly A. McClellan, Delphie Dugal-Tessier, Lisa M. Julian, Sonia P. Rodrigues, David S. Park, Timothy E. Kennedy, Ruth S. Slack

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APPENDIX C- FIRST AUTHOR REPRINTS

Brief Communications

The Retinoblastoma Protein Is Essential for Survival of Postmitotic Neurons

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Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

The retinoblastoma protein (Rb) family members are essential regulators of cell cycle progression, principally through regulation of the E2f transcription factors. Growing evidence indicates that abnormal cell cycle signals can participate in neuronal death. In this regard, the role of Rb (p105) itself has been controversial. Germline Rb deletion leads to massive neuronal loss, but initial reports argue that death is non-cell autonomous. To more definitively resolve this question, we generated acute murine knock-out models of Rb in terminally differentiated neurons *in vitro* and *in vivo*. Surprisingly, we report that acute inactivation of Rb in postmitotic neurons results in ectopic cell cycle protein expression and neuronal loss without concurrent induction of classical E2f-mediated apoptotic genes, such as Apaf1 or Puma. These results suggest that terminally differentiated neurons require Rb for continuous cell cycle repression and survival.

Introduction

The re-expression of cell cycle proteins in neurons, or cell cycle re-entry, has been observed in neurodegenerative conditions and injury such as stroke (Greene et al., 2007; Herrup and Yang, 2007; Rashidian et al., 2007). Recent experimental models implicate essential cell cycle regulators as critical upstream events in disease progression (Park et al., 1997b; Rashidian et al., 2005). For example, cyclin-dependent kinases (CDKs), key proteins involved in cell cycle progression, have been shown to regulate upstream events during neuronal loss in both *in vitro* and *in vivo* systems (Park et al., 1997b; Rashidian et al., 2005). In these cases, the pathogenic mechanism relating to reactivation of the cell cycle machinery has yet to be fully determined. The paradoxical case of cell cycle reactivation in a seemingly terminally quiescent neuronal population suggests the need for constant repressive action on cell cycle components.

The retinoblastoma protein (Rb), the first tumor suppressor identified, is best characterized for its role in regulating cell proliferation by the repression of E2f transcription factors (Burkhart and Sage, 2008). Upon proliferation-inducing stimulus, Rb is phosphorylated by CDK–cyclin complexes that disassociate the Rb/E2f complex allowing for the transcription of genes that initiate S-phase of the cell cycle (Burkhart and Sage, 2008). Rb has also been shown to regulate apoptosis by both direct and indirect mechanisms (Polager and Ginsberg, 2009). Rb targets include genes regulating cell cycle progression (*CyclinE1*, *CyclinA2*) (Burkhart and Sage, 2008), apoptosis (*Apaf1*, *Puma*, *SIVA*) (Polager and Ginsberg, 2009), and nonclassical functions (*Neogenin*, *Bnip3*) (Tracy et al., 2007; Andrusiak et al., 2011). Previous studies examining the role of cell cycle regulators in neuronal cell death observed that CDK inhibition, as well as Rb overexpression, are protective against apoptotic stimuli (Park et al., 1997a, 2000). Additionally, Rb is phosphorylated in a number of neuronal injury and degeneration models (Park et al., 2000; Biswas et al., 2007; Yu et al., 2012). These studies initially suggested that CDK-mediated inactivation of Rb may be critical in mediating neuronal loss; however, several pieces of evidence questioned this interpretation. First, while Rb germline knock-out mice showed massive neuronal loss, this was due to placental defects rather than neuron-specific pathways (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; de Bruin et al., 2003). Consistent with this, conditional Rb-deficient neural precursors divided ectopically, but were able to survive and differentiate into neurons (Ferguson et al., 2002; MacPherson et al., 2003). Whether this was due to compensation by the Rb homologs p107 and p130 is unknown. Second, previous studies implicated p130 and not Rb as the main mediator of neuronal death particularly in models of NGF withdrawal (Liu et al., 2005). Together, it poses the important question of whether Rb itself plays any significant role in neuronal cell cycle-mediated death.

Here, we sought to define the specific role for Rb in terminally differentiated postmitotic neurons. We show that Rb is essential to maintain survival of postmitotic neurons throughout adulthood. Rb loss induces expression of cell cycle regulatory genes; however, death following Rb loss appears to occur independently of transcriptional upregulation of apoptotic genes. This evidence demonstrates a crucial role for Rb in maintaining quiescence and survival of postmitotic neurons.

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Author contributions: M.G.A., R.V., and R.S.S. designed research; M.G.A. and R.V. performed research; M.G.A., R.V., D.S.P., and R.S.S. analyzed data; M.G.A., R.V., D.S.P., and R.S.S. wrote the paper.

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*M.G.A. and R.V. contributed equally to this work.

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Materials and Methods

Animals. All experiments were approved by the Animal Care Ethics Committee of the University of Ottawa and adhered to the *Guidelines of the Canadian Council on Animal Care*. Rb ^{flox} (Marino et al., 2000) and CamKCreERT2 (EMMA ID: 02125) (Erdmann et al., 2007) mice were maintained on FVBN and C57BL/6 backgrounds, respectively. Animals were genotyped according to standard protocols with previously published primers. All CamKCreERT2 animals used were heterozygotes for Cre expression. CamKCreERT2;Rb $^{flox/+}$ mice were crossed with Rb $^{flox/flox}$ mice to generate experimental animals, which at 5–6 weeks of age were given tamoxifen (TAM; Sigma) (180 mg/kg/d, i.p., for 5 d) and killed 1 or 4 weeks after the final injection. In all experiments, both female and male animals were used.

Primary cortical neurons. Embryonic cortical neurons were isolated by standard procedures (Fortin et al., 2001). Neurons were infected at the time of plating with a pWPXLD lentiviral vector expressing control GFP, GFP-tagged Cre recombinase, or dominant-negative DP1$_{\Delta 103-126}$ at a multiplicity of infection of 2. For immunofluorescence, cells were grown on coverslips and treated as indicated in the figure legends. Cells were then fixed with 4% PFA, and stained for Tuj1 (Covance), Ki67 (Cell Marque), γ-H2AX (Millipore), and DAPI. Alexa Fluor 488/594 secondary antibodies were used (Invitrogen). Statistical differences were determined using a one-way ANOVA, where $p < 0.05$ was considered statistically significant.

Western blots. Protein was isolated from cultured cortical neurons or cortical tissue, and Western blot analyses were performed as previously described (Ferguson et al., 2002) with antibodies directed toward activated caspase-3 (Cell Signaling Technology), Rb (PharMingen), CyclinA2 (Abcam), γ-H2AX (Millipore), and β-actin (Sigma).

Real-time PCR. Quantitative real-time PCR (qRT-PCR) assays were performed on primary cortical neurons using a Rotor-Gene RG-3000 (Corbett Research). Total RNA was isolated using Trizol method (Invitrogen) at 6 d *in vitro* (DIV). The SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) was used to amplify indicated target genes. All expression values were normalized to GAPDH. Primer sequences are available upon request. Expression values were obtained from three independent cultures, and significance was determined by a two-tailed Student's *t* test, where $p < 0.05$ was considered statistically significant.

Microarray. Rb $^{flox/flox}$ neurons were treated with GFP or Cre-GFP lentivirus and harvested after 6 DIV from three independent dissections. Total RNA was purified using Trizol method (Invitrogen). Samples were hybridized to Mouse Gene 1.0ST array (Affymetrix) at the Ottawa Hospital Research Institute Stemcore facility (Ontario, Canada). Data were normalized using the Robust Multichip Average expression measure (Irizarry et al., 2003), and statistically significant gene changes were determined using significance analysis of microrarrays (Tusher et al., 2001) in the TM4 MultiExperiment Viewer software package (Saeed et al., 2003). Significant changes in gene expression were computed using a fold increase of ≥1.5 and a false discovery rate (FDR) of <5%. Gene functional classification was performed using DAVID Bioinformatics (http://david.abcc.ncifcrf.gov/).

Tissue processing, immunohistochemistry, and cell quantification. Brains were perfused and fixed as previously described (Fortin et al., 2001). Sections were collected as 14 μm coronal cryosections on slides. For immunohistochemistry, sections underwent antigen retrieval in Target Retrieval Solution (Dako) and were incubated overnight at 4°C with the following primary antibodies: NeuN (Millipore), Ki67 (Cell Marque), and γ-H2AX (Millipore). Sections were incubated in blocking solution containing donkey anti-rabbit Alexa Fluor488 (Invitrogen) or donkey anti-mouse Cy3 (Jackson Immunoresearch) and DAPI. All images were acquired using a Zeiss 510 metaconfocal microscope under a 40× objective. For cell quantification, a minimum of three sections containing the frontal cortex was analyzed per brain. For each section, two fields in layer 2–4 and two fields in layer 5–6 were picked in the primary motor cortex, and the percentage of NeuN $^+$ cells among the total DAPI $^+$ cells, Ki67 $^+$ cells among NeuN $^+$ cells or γ-H2AX $^+$ cells among Ki67 $^+$ cells were quantified. Statistical analysis was determined by a two-tailed Student's *t* test, where $p < 0.05$ was considered statistically significant.

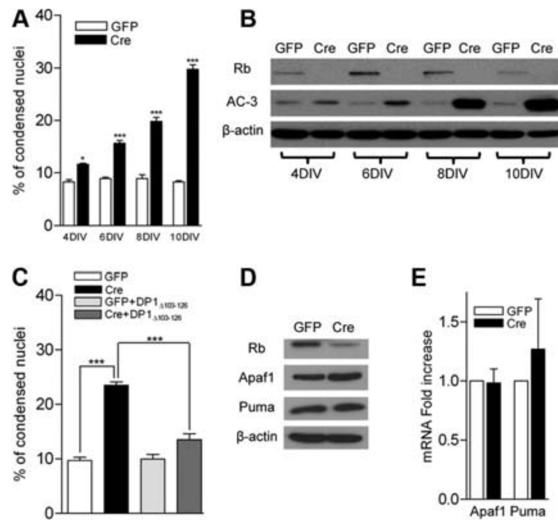
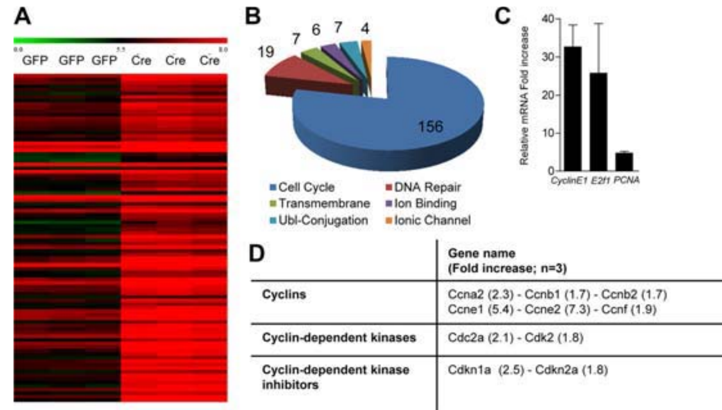


Figure 1. Acute Rb removal results in neuronal apoptosis independent of classical E2f-regulated apoptotic genes. ***A***, Cortical neurons were fixed at the indicated DIV and condensed nuclei were examined by DAPI staining ($n = 3$). ***B***, Western blot analysis of total protein extracted from control and Rb-deficient cortical neurons at the indicated DIV. ***C***, Cortical neurons were infected with the indicated constructs, and condensed nuclei were examined by DAPI staining at 8 DIV ($n = 3$). ***D***, Western blots on total protein extracted at 6 DIV. ***E***, qRT-PCR on RNA extracted at 6 DIV. RNA levels were normalized to GAPDH ($n = 3$). $^*p < 0.05$, $^{***}p < 0.001$. Error bars indicate SEM.

Results

Rb loss triggers apoptosis in primary cortical neurons

Previous evidence, as stated above, suggested that Rb does not regulate cell death in immature neural precursors. We were interested to see whether Rb may play a role in fully differentiated neurons. To accomplish this, we first established an acute paradigm in which we treated primary cortical neurons from animals harboring a conditional Rb allele (Rb $^{flox/flox}$) with lentiviruses expressing control-GFP (GFP) or Cre-GFP (Cre). Lentivirus carrying Cre was tested and had no toxic effects in wild-type neurons (data not shown). This system ablates Rb in cells infected with Cre virus (Fig. 1*B*). We examined neurons for induction of apoptosis by both microscopic assessment of condensed nuclei and Western blot analysis for activated caspase-3 (Fig. 1*A*,*B*). Rb loss was observed as early as 4 d after viral infection of Cre. Analysis of condensed nuclei by DAPI staining showed very little effect in Cre-infected neurons at 4 DIV; however, robust death was observed by 10 DIV (Fig. 1*A*). Activation of caspase-3 by Western blot analysis revealed an identical trend with caspase-3 activation peaking slightly earlier at 8 DIV (Fig. 1*B*). This evidence supports the notion that Rb plays an essential role in the survival of postmitotic neurons. To address whether the neuronal apoptosis we observe is E2f dependent, we broadly inhibited E2f activity using a dominant-negative DP1$_{\Delta 103-126}$ construct (Wu et al.,



	Gene name (Fold increase; n=3)
Cyclins	Ccna2 (2.3) - Ccnb1 (1.7) - Ccnb2 (1.7) Ccne1 (5.4) - Ccne2 (7.3) - Ccnf (1.9)
Cyclin-dependent kinases	Cdc2a (2.1) - Cdk2 (1.8)
Cyclin-dependent kinase inhibitors	Cdkn1a (2.5) - Cdkn2a (1.8)

Figure 2. Loss of Rb upregulates genes associated with cell cycle function. ***A***, Heat map representing top 200 genes significantly upregulated by microarray upon acute Rb deletion (fold increase of $\geq$1.5, FDR $<$5%, $n = 3$). ***B***, Gene functional classification of upregulated transcripts following Rb loss. ***C***, qRT-PCR validation of targets identified by microarray analysis. ***D***, Analysis of distribution of cell cycle regulatory genes induced upon Rb deletion. Error bars indicate SEM.

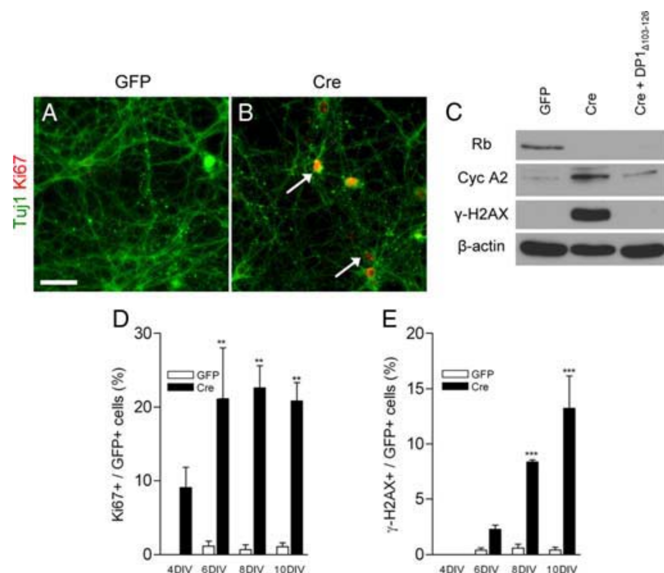


Figure 3. Rb-deficient neurons display cell cycle and DNA damage markers. ***A***, ***B***, Cortical neurons were fixed at 8 DIV and stained for Ki67. ***C***, Western blots were performed on total protein extracted at 8 DIV. ***D***, ***E***, Cortical neurons were fixed at the indicated DIV and quantified for the percentage of double labeling (Ki67$^+$/GFP$+$ over GFP$+$, γ-H2AX/GFP$+$ over GFP$+$) ($n =$ 3 or 4). $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar, 50 μm. Error bars indicate SEM.

1996). The inhibition of E2f activity was able to rescue the appearance of condensed nuclei upon Rb deletion, and had no deleterious effect on control neurons (Fig. 1*C*).

Rb loss does not induce classical E2f-regulated apoptotic genes

The Rb/E2f axis has been implicated in the direct transcriptional regulation of apoptotic machinery in a number of models (Polager and Ginsberg, 2009). The most well characterized gene targets consist of Apaf1 and the BH3-only protein Puma (Guo et al., 2001; Hershko and Ginsberg, 2004). We therefore asked the question of whether loss of Rb de-represses these apoptotic genes, thereby triggering a cell death cascade. We examined levels of the E2f-regulated apoptotic genes *Apaf1* and *Puma* by Western blot and qRT-PCR (Fig. 1*D*,*E*). We did not observe any significant upregulation of either gene at the level of protein or transcript, suggesting an alternative pathway of death.

Acute removal of Rb results in upregulation of cell cycle machinery

We next sought to identify the specific genes de-repressed upon loss of Rb in our primary cortical neurons. We used microarray to examine broad gene expression changes in response to Rb deletion. We examined neurons at 6 DIV as they do not yet display extensive apoptosis, which may result in indirect transcript dysregulation. Genes were deemed significant by significance analysis of microarray increase of $\geq$1.5-fold and a FDR of $\leq$5% ($n = 3$). We identified 377 increased transcripts upon Rb loss (Fig. 2*A*). Using DAVID Bioinformatics (http://david.abcc.ncifcrf.gov/), we used a functional classification of genes (Huang da W, et al., 2009) (Fig. 2*B*). Gene functional classification uses a modular enrichment analysis to assess term-to-term relationships to minimize redundancy in GO classification terms. Of the 377 genes identified as upregulated in our Rb-deficient neurons, 368 possessed DAVID IDs. Gene functional classification identified 14 gene clusters representing 216 genes. These clusters were manually reduced to Cell Cycle (156), DNA

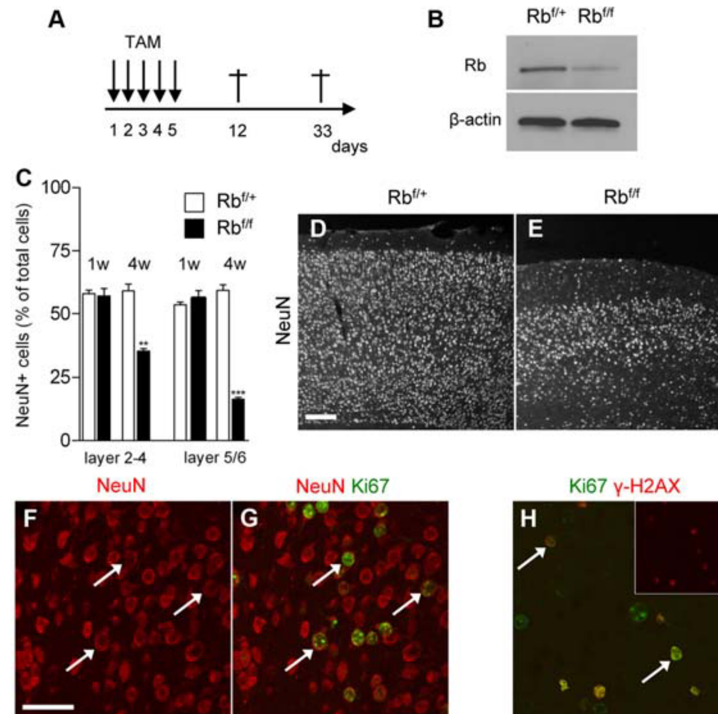


Figure 4. Acute Rb removal in adult neurons triggers neuronal loss. ***A***, TAM injection paradigm. ***B***, Western blot of cortex lysates confirming the decrease of Rb levels in CamKCreERT2; Rb$^{flox/flox}$mice (Rb$^{f/f}$) 1 week after the last TAM injection. ***C***, Rb loss results in neuronal loss in the cerebral cortex 4 weeks following TAM treatment ($n = 3$). ***D***, ***E***, Representative pictures of NeuN immunofluorescence displaying loss of neurons in the cortex of CamKCreERT2; Rb$^{flox/flox}$mice (***E***) compared with CamKCreERT2; Rb$^{flox/+}$ mice (***D***). ***F***, ***G***, Ectopic expression of the proliferative marker Ki67 in NeuN^{+} neurons (arrows) of CamKCreERT2; Rb$^{flox/flox}$ mice 1 week after the last TAM injection. ***H***, Ectopically proliferating Ki67^{+} cells exhibit γ-H2AX immunoreactivity (arrows) following Rb deficiency. $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bars: ***D***, ***E***, 100 μm; ***F–H***, 50 μm. Error bars indicate SEM.

Repair (19), Transmembrane (7), Ion Binding (6), Ubl-conjugation (7), and Ionic Channel (4) (Fig. 2*B*). This over-representation of cell cycle genes suggests a restricted role for Rb in postmitotic neurons in the maintenance of the quiescent state. Several genes including *PCNA* and *CyclinE1* were chosen for validation by qRT-PCR with upregulation on the array confirmed (Fig. 2*C*). Upregulated cell cycle genes were not confined to the G1/S transition, as factors associated with various facets of the cell cycle were induced (Fig. 2*D*). Our microarray data suggest that Rb plays a role in the regulation of specific gene classes that does not include classical E2f-regulated apoptotic genes in terminally differentiated neurons.

Acute removal of Rb triggers ectopic cell cycle protein expression and DNA damage

Our microarray analysis revealed a very specific cohort of genes related to cell cycle dynamics upregulated in the absence of Rb. We then asked whether upregulation of these genes would manifest itself as ectopic expression of cell cycle proteins. Rb$^{flox/flox}$ neurons infected with control and Cre lentivirus were fixed and stained for Tuj1 (neuronal marker) and Ki67 (proliferative marker). We observed Tuj1/Ki67^{+} very prominently in Rb-deficient neurons, which were rarely visible in control neurons (Fig. 3*A*,*B*). Neurons also exhibited an increase in protein levels of the known cell cycle regulatory protein CyclinA2 (Fig. 3*C*). Finally, we examined neurons for increased expression of γ-H2AX. γ-H2AX is the phosphorylated form of H2AX; this phosphorylation event is dependent upon induction of double strand breaks (DSBs) (Rogakou et al., 1998). We observed a significant increase in this mark in Rb-deficient neurons (Fig. 3*C*), suggesting that Rb loss triggers ectopic induction of cell cycle machinery and a DNA damage response. The induction of γ-H2AX and CyclinA2 is E2f dependent as introduction of $DP1_{\Delta 103-126}$ was able to rescue to control levels (Fig. 3*C*). To examine the temporal relationship between cell cycle re-entry and DNA damage, we immunostained for Ki67 and γ-H2AX at 4, 6, 8, and 10 DIV. We observed an increase in the number of Ki67^{+} cells upon Rb deletion as early as 4 DIV (Fig. 3*D*). The appearance of γ-H2AX was not apparent until 6 DIV, and increased at 8 and 10 DIV (Fig. 3*E*). These data suggest that double strand breaks occur downstream of cell cycle re-entry and that both of these processes are mediated by E2f activation.

Acute *in vivo* loss of Rb results in neurodegeneration

We finally asked whether conditional Rb loss would result in neurodegeneration of adult neurons *in vivo*. To this end, we used a TAM-inducible CamKCreERT2 mouse model to acutely delete Rb in adult forebrain neurons. Adult CamKCreERT2; Rb$^{flox/+}$ (Ctrl) and CamKCreERT2; Rb$^{flox/flox}$ mice (5–6 weeks of age)

were injected with TAM and killed 1 or 4 weeks after the last injection (Fig. 4*A*). Recombination efficiency was validated by Western blot (Fig. 4*B*). At 1 week postinjection, we did not detect any significant neuronal loss in the cerebral cortex of CamKCre-ERT2; $Rb^{flox/flox}$ animals (Fig. 4*C*). However, neuronal density was dramatically reduced in the cortex of knock-out animals 4 weeks after TAM induction (Fig. 4*C–E*). To determine whether Rb-deficient neurons ectopically express cell cycle proteins *in vivo*, we colabeled the neuronal marker NeuN with Ki67 at 1 week postinjection, before neuronal loss. We did not observe NeuN/$Ki67^+$ neurons in Ctrl animals; however, 22.5 ± 16.2% and 16.9 ± 3.9% ($n = 3$) of $NeuN^+$ neurons expressed Ki67 in the superficial (layers 2–4) and deep (layers 5–6) layers of Rb-deficient animals, respectively (Fig. 4*F*,*G*). Finally, a significant proportion of ectopic $Ki67^+$ cells in the cortex of Rb-deficient animals also exhibit γ-H2AX immunoreactivity (layer 2–4: 81.2 ± 7.3%; layer 5–6: 54.9 ± 5.1%; $n = 3$), indicating that Rb loss induces cell cycle proteins and DSB, which may contribute to neuronal loss *in vivo* (Fig. 4*H*).

Discussion

Our studies demonstrate several significant conclusions regarding neuronal survival. We have shown that acute loss of the cell cycle regulatory gene Rb in postmitotic neurons results in apoptosis. Critically, we show by microarray and candidate gene approaches that this neuronal loss is not due to de-repression of classical Rb/E2f-regulated apoptotic machinery. Instead, we observe the induction of mainly cell cycle- and DNA repair-related proteins. Finally, we provide *in vivo* evidence that acute Rb deletion is sufficient to induce neuronal loss. Together, these results highlight a specific role for Rb in the repression of cell cycle genes and survival in postmitotic neurons.

Importantly, our results highlight a differential dependence on Rb in proliferating neural precursors and terminally differentiated neurons. Germline Rb loss models displayed nonautonomous neuronal apoptosis, and *in vitro* differentiation of germline Rb-deficient precursors showcased the dispensable role for Rb at this stage of development (Callaghan et al., 1999). In more defined precursor-specific conditional deletion models, neuronal differentiation also proceeded in a largely normal fashion (Ferguson et al., 2002; MacPherson et al., 2003). In contrast, our present observations indicate that in terminally differentiated neurons, Rb is essential to maintain survival.

Our findings serve to further expand the role of the Rb family in the regulation of neuronal survival. In both steady-state and stress-induced paradigms, the Rb-related protein p130 was shown to be essential in the regulation of neuronal survival (Liu et al., 2005). Our data implicate Rb in a similar manner. It is important to note the latencies to cell death observed with either p130 or Rb loss. Upon siRNA-mediated p130 depletion, apoptosis was observed within 48 h (Liu et al., 2005), whereas, in our system Rb deletion results in similar levels of cell death after 120 h. This suggests that while both Rb and p130 may be essential in the maintenance of neuronal survival, the mechanisms used by each may differ. In neurons, p130 was shown to mediate gene repression via HDAC1-mediated interaction with Suv39h1-p130-E2f4 (Liu et al., 2005). The direct mechanisms that Rb employs to repress gene expression in neurons have yet to be fully described.

We and others have previously demonstrated a tight link between neuronal cell cycle re-entry and cell death. Importantly, our data emphasize that high E2f activity and induction of E2f target genes in the absence of Rb is deleterious for postmitotic neurons. However, the direct link between cell cycle re-entry and cell death remains correlative at this point, and future experiments will be required to determine exactly how cell cycle re-entry and DNA damage repair intersect to result in cell death in a postmitotic neuron. To our surprise, induction of cell death upon Rb deletion occurs independently of E2f-dependent transcriptional upregulation of classical Rb/E2f-mediated apoptotic factors such as Apaf1 and Puma. In this regard, comparison to other neuronal death paradigms involving abnormal cell cycle activation is informative. DNA damage-induced CDK activation, Rb phosphorylation, and subsequent cell loss occurs within hours of insult (Park et al., 2000). However, in this paradigm, independent activations of both CDK/Rb/E2f and p53 signals occur and work in concert to promote apoptosis (Morris et al., 2001). In contrast, our present data indicate that Rb deficiency alone results in a significantly protracted timing of death compared with DNA damage. This suggests that the efficiency and speed of death is regulated by the presence of multiple signals that act together to regulate apoptosis. In this regard, classical apoptotic targets like Apaf1 and Puma are known to be regulated by both E2fs and p53. Finally, it is interesting to note that while Rb loss itself induces markers of DNA damage and induction of DNA repair enzymes, the classical p53 response does not appear to be initially activated.

In conclusion, our results reveal an essential role for Rb in the maintenance of neuronal survival. Rb is able to actively repress genes that drive the cell cycle to prevent neurons from entering a proliferative state. Unlike other tissue systems, Rb loss does not trigger concurrent induction of classical E2f-regulated apoptotic targets. These findings reveal the need for constitutive Rb-mediated repression to prevent ectopic expression of cell cycle proteins and maintain survival in a terminally differentiated postmitotic neuron. It also highlights the differential role Rb plays in precursors compared with terminally differentiated neurons.

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Molecular and Cellular Biology

Rb/E2F Regulates Expression of Neogenin during Neuronal Migration

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Rb/E2F Regulates Expression of Neogenin during Neuronal Migration[▽]

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The Rb/E2F pathway has long been appreciated for its role in regulating cell cycle progression. Emerging evidence indicates that it also influences physiological events beyond regulation of the cell cycle. We have previously described a requirement for Rb/E2F mediating neuronal migration; however, the molecular mechanisms remain unknown, making this an ideal system to identify Rb/E2F-mediated atypical gene regulation *in vivo*. Here, we report that Rb regulates the expression of *neogenin*, a gene encoding a receptor involved in cell migration and axon guidance. Rb is capable of repressing E2F-mediated *neogenin* expression while E2F3 occupies a region containing E2F consensus sites on the *neogenin* promoter in native chromatin. Absence of Rb results in aberrant neuronal migration and adhesion in response to netrin-1, a known ligand for neogenin. Increased expression of neogenin through *ex vivo* electroporation results in impaired neuronal migration similar to that detected in forebrain-specific Rb deficiency. These findings show direct regulation of *neogenin* by the Rb/E2F pathway and demonstrate that regulation of *neogenin* expression is required for neural precursor migration. These studies identify a novel mechanism through which Rb regulates transcription of a gene beyond the classical E2F targets to regulate events distinct from cell cycle progression.

The Rb pathway is best characterized for its role in regulating cell cycle progression through E2F-mediated transcriptional regulation of classical cell cycle machinery target genes. Recently, however, accumulating *in vivo* and *in vitro* evidence is emerging to suggest that Rb and E2F are capable of regulating expression of atypical target genes with functions other than cell cycle regulation in cell-type-specific manners (reviewed in reference 35). *In vivo*, several studies have emerged that implicate Rb and E2F interaction in novel processes beyond well-characterized roles in cell cycle regulation (10; for a review, see reference 6). In the nervous system, in particular, we have recently shown that an Rb-E2F3 interaction mediates migration of a subpopulation of GABAergic interneurons (34). In the same study, we also observed deregulation of a number of genes with known roles in neuronal migration in cell populations lacking Rb, suggesting a role for E2F3 in regulating transcription of novel targets (34). A second cell cycle-independent role for E2F3a in regulating Rb-mediated interneuron differentiation was also reported in the retina (9). Thus far, *in vivo* studies have failed to identify the mechanism through which these cell cycle-independent processes occur.

In parallel, *in vitro* several microarray studies examining changes in gene expression in response to various models of deregulated E2F expression have each identified groups of overlapping novel target genes with well-characterized roles in differentiation, development, and migration (5, 15, 25, 31, 39, 41, 60). More recently, chromatin immunoprecipitation (ChIP)-on-chip studies have identified putative E2F binding sites within the promoters of a number of genes unrelated to the cell cycle (3, 4, 7, 28, 46, 56, 57). Finally, by using an approach whereby novel genes induced by E2F1 are identified based on subtraction screening, genes with known roles in differentiation and migration were identified as being directly induced by E2F1 in a cell cycle-independent manner (26). Thus, these data provide evidence that our understanding of the significance of Rb/E2F function should be expanded to include transcriptional regulation of genes beyond the well-characterized subset of targets that regulate the cell cycle.

Our identification of a role for Rb/E2F3 in mediating neuronal migration represents an attractive model to identify novel cell cycle-independent E2F target genes in the context of an *in vivo* physiological function (16, 34). Given our previous observations revealing (i) deregulation of a number of genes in families of known chemotactic ligands and receptors implicated in neuronal migration in the absence of Rb; and (ii) the cell-autonomous requirement for Rb in neuronal migration, we hypothesized that Rb/E2F may modulate the transcription of novel target genes involved in neuronal migration. We focused our efforts on *neogenin*, a receptor for the netrin and repulsive guidance molecule (RGM) families of chemotropic ligands (reviewed in reference 14). Notably, neogenin is highly expressed by a subpopulation of interneurons migrating from the ventral forebrain and has been independently identified, in an *in vitro* overexpression system, as an E2F-regulated gene (26, 34). Here, we report that Rb directly regulates the expression of a nontraditional target, *neogenin*. Rb is capable of repressing E2F-mediated transcription of *neogenin* while E2F3 binds to a region containing a conserved E2F consensus site on

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the *neogenin* promoter in native chromatin. The absence of Rb results in aberrant neuronal migration and adhesion in response to the neogenin ligand, netrin-1. Finally, increased expression of neogenin through *ex vivo* electroporation results in impaired neural precursor migration similar to that observed in forebrain-specific Rb deficiency. From these findings, we show direct regulation of *neogenin* by the Rb/E2F pathway and demonstrate that correct regulation of *neogenin* expression is required for neural precursor cell migration. Through these studies we identify a novel mechanism through which Rb interacts with E2F to regulate transcription of genes beyond the classical E2F targets to influence biologically relevant events distinct from cell cycle progression.

MATERIALS AND METHODS

Mice. Telencephalon-specific Rb-deficient mice were generated by crossing floxed Rb-F19 (33, 53) and Foxg1-cre mice (23), and mice were genotyped according to standard protocols with previously published primers (16, 17). For embryonic time points, the time of plug identification was counted as embryonic day 0.5 (E0.5). For all experiments littermate Rb conditional mutants ($Rb^{flox/flox}$ Foxg1-$cre^{+/-}$) and double heterozygous controls ($Rb^{flox/+}$ Foxg1-$cre^{+/-}$) were compared. Due to Rb autoregulation (49), Rb expression in heterozygous mice is similar to that of wild-type controls. All experiments were approved by the University of Ottawa's Animal Care ethics committee, adhering to the Guidelines of the Canadian Council on Animal Care.

Western blotting. Protein was isolated from neurospheres by treating cells with lysis buffer (10 mM Tris, 0.15 M NaCl, 1 mM EDTA, 0.4 mM sodium vanadate, and 0.5% Triton-X). Cells were incubated on ice for 20 min, followed by a 10-min centrifugation at maximum speed to remove debris. Western blotting was performed as previously described (16) with antibodies directed toward neogenin (H-175; Santa Cruz), Rb (Pharmingen), and beta-actin (Sigma). Immunoblotting was performed on three independent samples, and results were quantified using ImageJ software.

Tissue preparation and *in situ* hybridization. Tissue was dissected, fixed, cryoprotected, and sectioned as previously described (34). Nonradioactive *in situ* hybridization and digoxigenin probe labeling were performed according to previously described protocols (54). Neogenin, deleted in colorectal cancer (DCC), netrin, and RGMa, were generous gifts of Helen Cooper of the University of Queensland (20), Elke Stein of Yale University, and Silvia Arber of the University of Basel (40). All results shown are representative of those obtained with a minimum of three independent animals.

Transcription factor binding sites. The 5′ region of the mouse Neo1 locus containing the intergenic region, the untranslated region, and exon 1 was analyzed for putative E2F binding sites using the TRANSFAC Professional Library, version 10.2, through Mulan/MultiTF (http://rvista.dcode.org). All sites identified in Mulan were manually examined for their similarity to the consensus (TTTSSCGC) and nonconsensus (BKTSSCGS) E2F motifs.

Chromatin immunoprecipitation. Neurosphere cultures were prepared from CD1 embryos (Charles River) at embryonic day 14.5. Proliferating neurospheres were triturated, cross-linked with formaldehyde, lysed, sonicated, and centrifuged at 14,000 × *g* to remove cellular debris. Each immunoprecipitation was performed using 2 μg of antibody. Antibodies against E2F3 (sc-878), E2F1 (sc-193), and normal rabbit immunoglobulin G (sc-2027) were obtained from Santa Cruz Biotechnology. Immunocomplexes were captured using protein A/G9-Sepharose beads and washed extensively, and cross-links were reversed overnight, followed by treatment with RNase A at 37°C for 1 h and proteinase K at 65°C for 30 min. The purified DNA was examined by PCR using primers designed around the E2F consensus sites at bp −44 and bp −821 in the 5′ region of the neogenin gene. Immunoprecipitations were performed from three cultures obtained from three independent animals.

DNA constructs. The *neogenin* construct was PCR amplified from pSecTagA-Neogenin (rat) using the following primers: forward, GAACTGCAGACCATG GAAGAAAGA; reverse, CTTCTGCAGGTTGCCCTCTAGCTAG. The PCR fragment was then subcloned into pCIG2. The 5′ regulatory region of *neogenin* was PCR amplified from embryonic genomic DNA. Primers were designed as follows with flanking XhoI restriction sites inserted: full-length forward, AGAC TCGAGGAGGTGCAGAGGAGTCGC; full-length reverse, TGTCTCGAGG TTGAAAAACCAATTCCCG. To create 5′ truncations the following primers were used with full-length reverse primer: FTrunc247, AGACTCGAGAGCCG GGGGGTGG; FTrunc618, AGACTCGAGAAGCGATCCGCCTCCT. To create the 3′ truncation the following primer was used with full-length forward primer: RTrunc 247np, TGTCTCGAGCCACCCCCCGGCT. The construct consisting of bp 247 to 618 was made using the FTrunc247 and RTrunc 618bp (TGTCTCGAGGCGGATCGCTTCTCC) primers. PCR products were digested with XhoI and ligated into pGL4.24 (Promega). Sequence was confirmed by DNA sequencing (StemCore Laboratories, University of Ottawa).

Luciferase reporter assays. HEK293T cells were transfected using Lipofectamine (Invitrogen) as per the manufacturer's protocol. Briefly, cells were transfected with 500 ng of pGL4.24, pGL4.24-Neogenin, or pGL4.24-NeoTruncations, 10 ng of E2F3, 300 ng of Rb, and total transfected plasmid normalized with pcDNA3.1. Transfection efficiency was normalized using 10 ng of pRL *Renilla*-expressing vector. Cells were lysed at 24 h posttransfection and examined by spectrophotometer (LMaxII; Molecular Devices) for luciferase expression by a Dual-Glo luciferase kit (Promega).

In vitro explant cultures. *In vitro* explant cultures were performed as described previously (13, 29, 38, 43, 44) with some modifications. Briefly, brains were removed from E14.5 embryos in L-15 (Gibco) medium, and medial ganglionic eminence (MGE) was dissected as described previously (16); explants were subsequently divided into pieces approximately 200 μm in diameter by using sharpened tungsten needles. MGE explants were then transferred into collagen (PureCol, catalog number 5409; Inamed BioMaterials) inside culture dishes and allowed to solidify for 40 min prior to addition of Neurobasal medium (Gibco) supplemented with fetal bovine serum (FBS). Purified netrin-1 was added to the explant culture medium at a final concentration of 200 ng/ml.

Explants cultured alone were grown *in vitro* for 24 h. Images were captured with a Zeiss Axioscope microscope. For quantification of cell migration in collagen, the total number of cell bodies migrating from explants was counted. Two to four explants per embryo were measured, and values were averaged. Two-tailed *t* tests were performed to compare mean migration between genotypes or treatment groups. Differences were considered significant at a P value of <0.05.

Neural progenitor cultures. Pregnant mice were euthanized at gestation day 14.5, embryos were removed, and the ganglionic eminences were isolated by microdissection. For determination of cell proliferation and cell death in single-cell preparations, ganglionic eminences were dissociated, and equal cell numbers were plated on poly-D-lysine- and laminin-1-coated dishes in duplicate. Cells were cultured in Neurobasal medium supplemented with 0.5 mM L-glutamine, 1% N-2, 2% B-27, 10 ng/ml fibroblast growth factor 2 (FGF-2), and 20 ng/ml epidermal growth factor (EGF) in either the presence or absence of netrin-1 at 200 ng/ml. Cells were treated with bromodeoxyuridine (BrdU) at a final concentration of 10 μg/ml for 45 min prior to fixation. After 24 h, cells were fixed for 15 min in 4% paraformaldehyde (PFA) and then treated sequentially with 2 N HCl and 0.01 M NaB_4O_7, followed by BrdU immunohistochemistry (anti-BrdU at 1:100; BD Biosciences, San Jose, CA) and Hoechst nuclear staining. The total cells and BrdU-positive cells were counted in three microscope fields per duplicate well. Rates of proliferation were obtained by calculating the proportion of BrdU-positive cells relative to the total cell number. Fold increase in proliferation in response to netrin-1 was calculated for each genotype by dividing the percentage of proliferating cells in the presence of netrin-1 by the percentage of proliferating cells in the absence of netrin-1. (Three separate embryos were analyzed in quadruplicate for both control and conditional mutant embryos). For Hoechst labeling, dead cells were identified by the characteristic condensation of chromatin. Fold increase in apoptotic nuclei was calculated in an analogous manner to cell proliferation. (Three separate embryos were analyzed in quadruplicate for both control and conditional mutant embryos).

Substrate-bound adhesion assay. To assess cell adhesion in the presence of netrin-1, a substrate-bound adhesion assay was performed as described previously (50). Briefly, 20 μl of 0.1% nitrocellulose (Hybond ECL; Amersham Biosciences) dissolved in methanol was dried on the bottom of a four-well plate. Plates were then incubated with either Hanks' balanced salt solution (HBSS) or 2 μg/ml netrin-1 in HBSS for 2 h at room temperature. Wells were then blocked for 1 h with 1% bovine serum albumin (BSA; Fisher Scientific) in HBSS and then again with 1% heparin (Sigma) in HBSS. A total of 2.5×10^5 cells from dissociated ganglionic eminences were plated in Neurobasal medium (Gibco) supplemented with 2% B-27 and 2 mM glutamine. Cells were cultured for 2 h at 37°C in 5% CO_2, gently washed once with phosphate-buffered saline (PBS), and fixed with 4% PFA in PBS overnight. Nuclei were labeled with 0.5 μg/ml Hoechst 33258 (Sigma) in PBS for 30 min. Experiments were performed on four wild-type and three mutant embryos. Paired, two-tailed *t* tests were performed to compare genotypes, with differences considered significant at a P value of <0.05.

***Ex vivo* cortical electroporation.** Cortical electroporation and *ex vivo* slice culture were performed as described previously (16, 22, 42), with some modifications. Briefly, pregnant female mice (Charles River) were euthanized at E15

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with a lethal injection of sodium pentobarbital. Embryos were removed and decapitated, and a 2 μg/μl solution of pCIG-Neogenin or empty pCIG vector (supplemented with 0.5% Brilliant Blue FCF for visualization) was injected, using a Picospritzer II (General Valve Corporation), into the lateral ventricles. Brains were subjected to 10 pulses at 70 V using an ElectroSquarePorator ECM830 (BTX/Genetronics San Diego, CA). Brains were then isolated and embedded in low-melting-point agarose. Agarose-embedded brains were sectioned coronally into 250-μm sections on a Leica VT1000S vibratome. Brain sections were collected and plated on poly-L-lysine–laminin-coated filter membrane inserts placed on top of the culture medium in each well of a six-well dish, as described previously (16, 42). Slices were then cultured for 72 h to assess the degree of migration. Two sections from five to six embryos from three litters were measured, and values were averaged. Migration was assessed by measuring the total area occupied by green fluorescent protein (GFP)-positive cells at both 24-h and 72-h time points. Degree of migration under each condition was assessed by subtracting the area occupied at 24 h from the area occupied at 72 h and then dividing this value by the initial area to obtain a percentage of migration over time. Degree of migration in neogenin-expressing samples relative to the control was obtained by dividing percent migration in neogenin by percent migration of the control. Two-tailed *t* tests were performed to compare migration between control and neogenin overexpression, with differences considered significant at a *P* value of <0.05.

RESULTS

Rb deficiency results in specific deregulation of *neogenin* expression *in vivo*. Our previous studies have described a physiological requirement for Rb interacting through E2F to mediate nervous system development (34). Furthermore, we reported that Rb deficiency results in increased expression of mRNA encoding neogenin, a receptor involved in regulating axon guidance and cell migration during neural development (59; for a review, see reference 14).The physiological significance of Rb-mediated regulation of *neogenin* expression on nervous system development, however, is unknown. To assess the contribution of neogenin to Rb-mediated nervous system development, we first asked if deregulation is unique to *neogenin* or extends across the family of neogenin ligands and related receptors. To address this question in the telencephalon, sections from Foxg1-cre conditional Rb mutants were subjected to *in situ* hybridization to examine the expression profiles of neogenin, the closely related receptor deleted in colorectal cancer (DCC), and the known neogenin ligands repulsive guidance molecule (RGMa) and netrin-1 (Fig. 1A). Consistent with our previous microarray and *in situ* hybridization findings (34), we detected increased neogenin expression throughout the ventral and dorsal telencephalon in conditional Rb mutants. In the ganglionic eminence, a source of migrating interneurons, no difference was detected in the expression patterns of DCC, netrin-1, and RGMa between control and conditional Rb mutants ($n = 3$) (Fig. 1A). These expression patterns parallel results of other studies that have shown neogenin and netrin-1 overlapping protein expression in the ganglionic eminence (19, 52). Thus, of the members of the netrin signaling pathway, a significant change was detected in the expression only of neogenin within the ventrally derived population of neural precursor cells. To validate increased neogenin levels identified by *in situ* hybridization, we assessed expression at the protein level within the migrating cell population. Total protein was extracted from the population of ventrally derived neural progenitor cells from three separate embryos for each genotype, and a similar increase in neogenin protein was identified in conditional Rb mutants (Fig. 1B). Efficient excision of the Rb allele in the context of primary

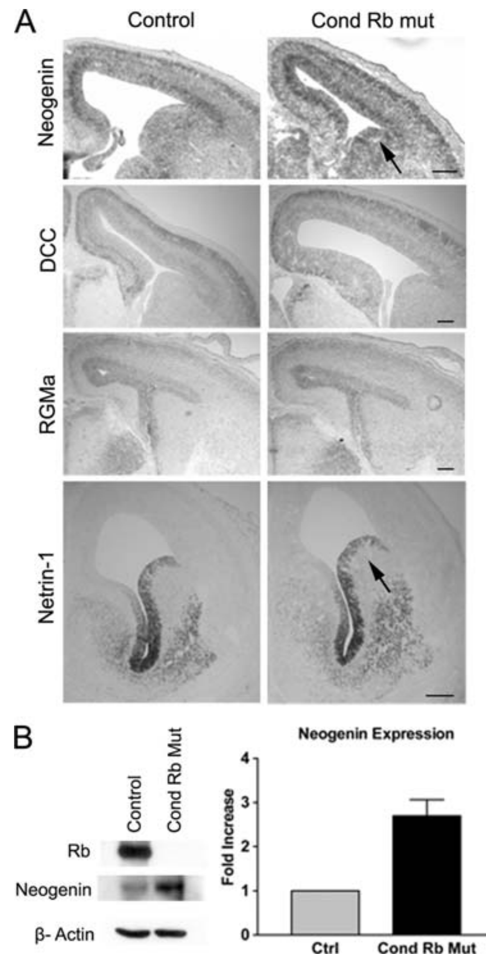

FIG. 1. Neogenin is upregulated in the absence of Rb in the developing forebrain. (A) In the absence of Rb (*Foxg1-Cre*/+; $Rb^{loxP/loxP}$) increased neogenin expression is detected in the subventricular zone, cortex, and striatum compared with results in controls (*Foxg1-Cre*/+; $Rb^{loxP/+}$). *In situ* hybridizations were performed on three or more independent samples for DCC, RGMa, netrin-1, and neogenin. Arrows indicate regions of overlapping expression between neogenin and netrin-1 in the ganglionic eminence. (B) Western blot analysis was performed on neural precursor cells isolated from E14.5 Rb conditional mutants and control embryos. Note efficient recombination of the floxed Rb allele, showing no detectable Rb. Rb mutants showed an upregulation of neogenin compared to levels in the control. Densitometric quantification of neogenin expression detected by Western blotting was performed using ImageJ analysis of three independent experiments. Significance was determined through a paired two-tailed *t* test for the control and conditional Rb mutant ($P < 0.05$).

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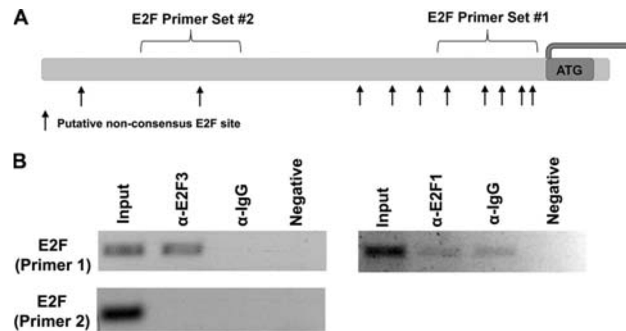

FIG. 2. E2F3 interacts with a region containing multiple putative E2F sites within the 5′ regulatory region of neogenin. (A) Putative E2F sites were identified in the 5′ region of the mouse (mm9) Neo1 gene using Mulan/rVista software and confirmed by manual sequence analysis (BKTSSCGS). (B) ChIP was performed on neurospheres isolated from the ganglionic eminence of E14.5 wild-type embryos. Immunoprecipitation was performed using an antibody specific to E2F3 or E2F1, followed by PCR amplification of the indicated regions in the 5′ regulatory region of the neogenin gene. ChIP was performed on three independent cultures per condition. An interaction was detected only with E2F3 at the region containing multiple clustered sites; the putative site at 852 bp showed no specific binding. Immunoprecipitations were performed on independent cultures from three animals.

ventral neural precursors was confirmed by protein levels (Fig. 1B). Together, these data support the hypothesis that Rb may play an important role in regulating expression of neogenin, a nontraditional E2F target gene.

E2F3 binds the 5′ locus of neogenin *in vivo*. Recent studies have reported that *neogenin* is among a novel class of atypical E2F target genes regulated in a cell cycle-independent manner (26). E2F1 was shown to be capable of directly inducing neogenin expression independent of growth stimulation; however, no classical E2F binding (TTTSSCGC) site was observed within the 5′ region (26). With the implementation of new bioinformatic techniques, a new broad E2F consensus binding site(s) (BKTSSCGS) has recently been characterized (45). We asked whether E2F could be mediating neogenin expression through one of these newly defined E2F sites contained in its 5′ regulatory region. Using Mulan/rVista software, the 5′ region of the mouse *neogenin* gene was examined for the presence of broad-spectrum BKTSSCGS E2F motifs (45). Consistent with previous reports, no classical E2F binding sites could be found in the mouse *neogenin* promoter (26). Closer examination revealed 10 broad E2F binding sites located within 1.23 kb upstream of the translational start site. The majority of these putative E2F sites are clustered within the first 600 bp upstream of the ATG (Fig. 2A).

Prior studies examined the regulation of neogenin expression by E2F1; however, as we have previously described unique roles for E2F3 in nervous system development (34, 36), we asked whether E2F3 interacted with the 5′ regulatory region of the *neogenin* gene in the context of native chromatin. To address this question, we performed chromatin immunoprecipitation (ChIP) in primary neural progenitor cultures to see if E2F3 associated with the regions containing atypical E2F sites. Chromatin was immunoprecipitated with antibodies to E2F3, followed by PCR with primers designed around the cluster of E2F sites immediately upstream of the translation start and the region containing only a single site 852 bp upstream (Fig. 2). ChIP with the primer set surrounding the cluster demonstrated enrichment of E2F3 binding, with no detectable E2F3 binding at the site at −852 bp (Fig. 2B). Consistent results were obtained from three independent primary cultures. Previous studies using a subtractive microarray analysis revealed that E2F1 could induce neogenin expression in rat embryonic fibroblasts; however, a direct interaction in the *neogenin* regulatory regions was not shown (26). We therefore asked if E2F1 might physically interact with the putative promoter region of *neogenin*. To this end we performed ChIP with an antibody directed to E2F1 and examined the region in which E2F3 binding was detected. No enrichment was found in this region (Fig. 2B), suggesting that E2F1 does not interact with the *neogenin* promoter in precursors from the ventral forebrain. These results demonstrate that, in the context of neural precursor cells, E2F3 specifically binds the *neogenin* promoter at the region (−600 to ATG) encompassing the multiple putative E2F consensus sites in an *in vivo* context, and this is consistent with the hypothesis that E2F3 is capable of modulating neogenin gene expression.

Rb/E2F regulates transcriptional activity at the 5′ neogenin-regulatory region. Given that we observe deregulated neogenin expression in the absence of Rb, we next asked whether Rb regulates this expression through its interaction with E2F. To address this question, we performed *in vitro* luciferase reporter assays in HEK293T cells. Ideally, these studies should be performed in primary systems; however, overexpression of E2F1 or E2F3 induces a rapid and robust apoptotic response in embryonic tissue and in primary neural precursor cells. As HEK293T cell lines express E1B preventing apoptosis (58), they withstand overexpression of "activating" E2F constructs without undergoing cell death. Thus, all reporter assays were performed in HEK293T cell lines. The *neogenin* promoter region was amplified from embryonic genomic DNA with primers designed to flank a 1.23-kb region containing the putative E2F binding sites (Fig. 3A). The fragment was then subcloned into a luciferase reporter vector (pGL4.24) which was subsequently transfected into HEK293 cells.

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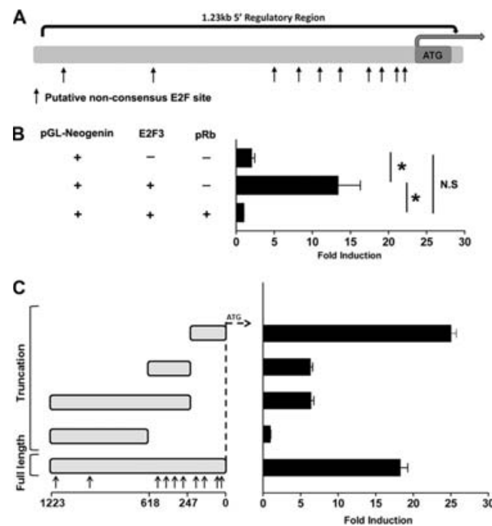

FIG. 3. The 5′ neogenin promoter is responsive to Rb/E2F regulation. (A) Schematic of the 5′ region of the neogenin gene. The 1.23-kb region isolated and cloned into the pGL4.24 vector contains 10 putative E2F binding sites identified using Dcode/Mulan software. (B) Dual-Glo luciferase (Promega) promoter assays in HEK293T cells utilizing the neogenin promoter reveal that the addition of E2F3 induces a 13-fold induction of neogenin promoter activity. When cells are stimulated with Rb and E2F3, the activation is eliminated, and luciferase levels return to that of promoter alone. (C) Luciferase analysis of truncations of the 5′ region of the neogenin gene. Activity is ablated upon removal of the initial 618 bp. The region between bp 247 and 618 results in slight E2F3 responsiveness; however, the first 247 bp recapitulates full-length promoter activity. *, $P < 0.05$; N.S., nonsignificant difference.

In the absence of exogenous E2F, the *neogenin* promoter displayed a minimal level of activation (Fig. 3B). Upon addition of E2F3, however, we observed a strong 13.3-fold increase in luciferase activity, demonstrating that E2F3 is capable of transcriptionally activating the *neogenin* promoter. We next asked if Rb is capable of repressing E2F-mediated activation of the *neogenin* promoter. Upon cotransfection of E2F3 and Rb expression plasmids, E2F3-mediated activation of the *neogenin* promoter was repressed back to basal levels (Fig. 3B). Together these results demonstrate that Rb acts to repress E2F3-mediated activation of the *neogenin* promoter through interaction in the 5′ regulatory region.

We next sought to determine the specific region in the neogenin promoter in which E2F3 was binding to activate transcription. Examination of the 5′-proximal promoter for both classical/nonclassical E2F binding motifs revealed 10 putative sites within the 1.23 kb examined. To define the essential regions required for E2F-mediated activation, we created multiple truncation constructs at roughly 250-bp intervals from both the 5′ and 3′ ends of the 1.23-kb promoter construct (Fig. 3C). While constructs lacking the regions upstream of bp −247 had no effect on E2F responsiveness, absence of the region from the ATG to bp −618 abolished the ability of E2F to activate transcription (Fig. 3C). This region was most enriched with eight putative E2F sites, and the results suggest that E2F3 is binding one or more of the several clustered sites in this region. To more precisely identify the sites, two further truncation constructs (bp 0 to 247 and bp 247 to 618) were made lacking each of the two clusters of E2F sites within the first 618 bp of the promoter (Fig. 3C). Introduction of the construct containing only the region encompassing bp 247 to 618 upstream of the promoter (Fig. 3C) resulted in slight activation in response to E2F (Fig. 3C). This result recapitulated the slight E2F responsiveness observed in the construct lacking the first 247 bp upstream of the ATG. To determine if the first 247 bp could confer E2F-mediated activation, this 247-bp fragment alone was ligated into the reporter construct, and full E2F responsiveness, equivalent to that of the full-length construct, was obtained. These results demonstrate that the cluster of E2F sites contained in the 247 bp upstream of the ATG are essential for neogenin promoter activation, consistent with the region identified by our E2F3 ChIP (Fig. 2B). These findings support the conclusion that Rb, acting through E2F3, directs the expression of neogenin, an atypical E2F target gene, that functions outside cell cycle progression.

Rb deficiency results in aberrant neuronal migration in the presence of netrin-1. We next sought to determine the functional consequences of deregulation of *neogenin* expression as a result of the ablation of the Rb gene. We hypothesized that if deregulated expression of *neogenin* contributes to the aberrant migration of ventrally derived neurons in the conditional Rb mutant, then neural precursor cells should elicit an aberrant response in the presence of neogenin ligand. During mammary gland development, netrin-1–neogenin interactions have been shown to be crucial for proper stabilization of the multipotent progenitor cell layer (19, 51). This interaction may play an analogous role during tangential migration in the developing forebrain. We therefore determined if netrin-1 is capable of influencing migration of MGE-derived cells under wild-type conditions. To effectively address this question, we employed a reductionist *in vitro* approach using primary neural precursor explants cultured in a collagen matrix. This approach created a defined extracellular environment containing netrin-1 alone and allowed us to determine the effect of the neogenin ligand, netrin, in the absence of other competing signals known to influence migration (32). Explants of ventral ganglionic eminence were microdissected from control and mutant E14.5 cerebral hemispheres and then cultured for 24 h supplemented with netrin-1, after which cell migration from the explant was quantified. We assessed the relative contribution of netrin-1, a ligand demonstrated to elicit neogenin-dependent chemoattractant responses in the developing nervous system. Explants were cultured in collagen, a matrix suitable for assessing chemoattractant responses (29, 38). Both control and conditional Rb mutant explants cultured in collagen alone exhibited modest numbers of cells migrating, with no appreciable difference in migration from either type of explant. In the presence of netrin-1, however, a clear difference was observed (Fig. 4A). While control explants exhibited a 4-fold increase in migration in the presence of netrin-1, there was no significant difference in the number of cells migrating from

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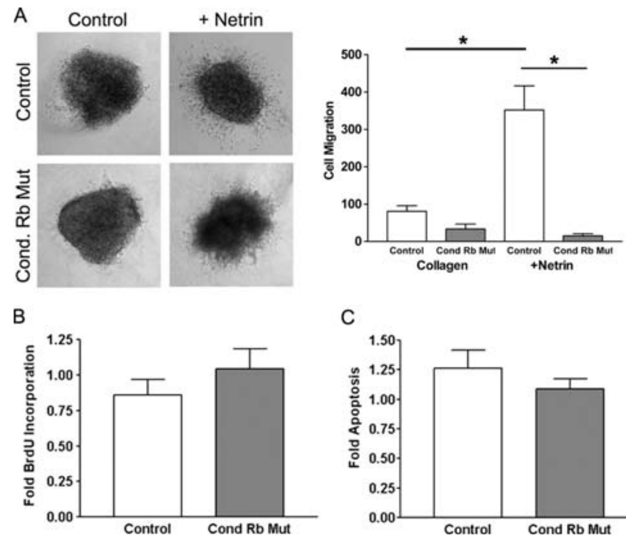

FIG. 4. Conditional Rb mutants display a defective migratory response to netrin-1. (A) Control (*Foxg1-Cre*/+; $Rb^{loxP/+}$) and conditional Rb mutant (*Foxg1-Cre*/+; $Rb^{loxP/loxP}$) MGE explants were cultured in collagen in the absence or presence of recombinant netrin-1. Migration was quantified by counting the individual cell bodies migrating from each explant. Bars in the graph at left represent the mean of the average number of cells migrating from an individual explant ± standard error of the mean. While control cells exhibit a nearly 4-fold increase in migration in the presence of netrin, no difference was observed in conditional Rb mutants between the presence and absence of netrin. Significance was determined through a paired two-tailed *t* test for explants of the same genotype and a two-tailed *t* test for explants of different genotypes. *, $P < 0.05$ ($n = 4$ embryos per treatment, per genotype; two to three explants were examined per embryo). (B and C) Cells from the ganglionic eminence of control and conditional Rb mutants were dissected and cultured as single-cell preparations in the presence or absence of netrin-1. Quantification of the proportion of cells in S phase (BrdU) or dying (Hoechst) reveals no change upon addition of netrin-1 in either genotype. Three separate embryos were analyzed in quadruplicate for both the control and conditional Rb mutant.

conditional Rb mutant explants cultured in the presence or absence of netrin-1 (Fig. 4A).

Netrin-1 has been hypothesized to mediate cell proliferation and cell death (37; for a review, see reference 11). We therefore verified that the differences detected in migration were not due to either of these processes. To dissect the potential contribution of altered proliferation or cell death, netrin-1 treatment was performed under conditions that recapitulate those used in our *in vitro* explant culture in order to ensure that there are no changes in proliferation and cell death under those specific conditions. To examine proliferation, cultures were treated with bromodeoxyuridine (BrdU), and the proportion of cells in S phase of the cell cycle was counted. In three independent control and mutant cultures, netrin-1 treatment did not significantly impact the number of proliferating cells (Fig. 4B). Assessment of chromatin condensation revealed no significant change in cell death between control and mutant cultures upon addition of netrin-1 ($n = 3$) (Fig. 4C). These results suggest that the increased number of cells migrating in response to netrin-1 is not a consequence of increased cell proliferation, nor can the absence of migration in the conditional Rb mutant be attributed to increased cell death. Thus, these data support a model whereby netrin-1 is capable of influencing migration of ventrally derived progenitors, an effect that is not observed in the conditional Rb mutants. These results suggest that ventrally derived progenitors from Rb mutants are inherently unable to elicit the appropriate migratory response to netrin-1 itself.

Increased adhesion to substrate-bound netrin-1 in conditional Rb mutants. A previous study demonstrated that netrin-1 and neogenin interact to mediate adhesion in the mammary gland (51). Given that we observe reduced migration in response to netrin-1 in the conditional Rb mutant, where *neogenin* expression is increased, we determined if the increased amount of neogenin present would increase adhesion of neural precursor cells. To address this, adhesion assays were performed which have been previously used to assess netrin-neogenin-mediated adhesion in fibroblasts (51) and adapted the assay for neural precursor cells (50). Using this assay, we examined the capacity of ventrally derived precursors from control and conditional Rb mutants to adhere specifically to immobilized netrin-1. The ventral telencephalon from E14.5 control and conditional Rb mutants was dissected and dissociated into single-cell suspensions. Cells were quantified, and then equal numbers were plated and allowed to adhere to culture dishes preadsorbed with nitrocellulose alone or with netrin-1 and nitrocellulose. After 2 h, cells were washed and fixed, and cell adhesion was quantified. Data were represented as the fold increase in adhesion upon netrin-1 treatment to eliminate the experimental variability observed from each in-

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TABLE 1. Rb-deficient neural precursors show an increased propensity to adhere to substrate bound netrin-1

Expt[a]	Embryo	Genotype	Cell adhesion (avg no. of cells/field)[b]		Fold increase[c]
			Without netrin	With netrin	
CTL	MGA57	*Foxg1-Cre*/+; $Rb^{loxP/+}$	382	925	2.421466
CTL	MGA62	*Foxg1-Cre*/+; $Rb^{loxP/+}$	235.5	513	2.178344
CTL	MGA132	*Foxg1-Cre*/+; $Rb^{loxP/+}$	121.5	285.5	2.349794
CTL	MGA133	*Foxg1-Cre*/+; $Rb^{loxP/+}$	127.75	291.4	2.281018
$Rb^{-/-}$	MGA134	*Foxg1-Cre*/+; Rb^{loxP}/Rb^{loxP}	100	316.25	3.1625
$Rb^{-/-}$	MGA136	*Foxg1-Cre*/+; Rb^{loxP}/Rb^{loxP}	113.5	504.75	4.447137
$Rb^{-/-}$	MGA59	*Foxg1-Cre*/+; Rb^{loxP}/Rb^{loxP}	464	1635	3.523707

[a] CTL, control.
[b] Cells from the ganglionic eminence of control and conditional Rb mutants were dissected at E14.5. Cells were allowed to adhere to netrin-1 or noncoated wells for 2 h before they were fixed and stained. Cells were then imaged, and nuclei were counted. The total number of nuclei per field was averaged for each condition. In the absence of Rb, cells from the ganglionic eminence display more significant adherence to netrin-1 than cells from control littermates.
[c] Fold increase represents the increase in adhesion from noncoated to netrin-1-coated wells.

dependent assay. Independent experiments, however, produced highly consistent results (Table 1). In the presence of netrin-1, conditional Rb mutants displayed a 3.6-fold increase in adhesion, whereas control littermates displayed a significantly smaller 2.5-fold increase (Fig. 5). Our findings suggest

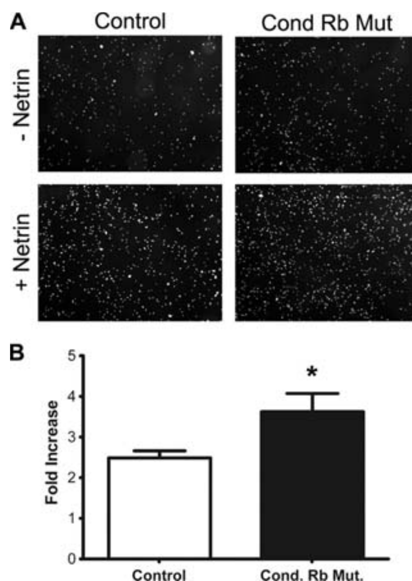

FIG. 5. Rb-deficient neural precursors show an increased propensity to adhere to substrate-bound netrin-1. (A) Cells from the ganglionic eminence of control (*Foxg1-Cre*/+; $Rb^{loxP/+}$) and conditional Rb mutants (*Foxg1-Cre*/+; $Rb^{loxP/loxP}$) were dissected at E14.5. Cells were allowed to adhere to netrin-1 or noncoated wells for 2 h and were then fixed and stained. Cells were then imaged, and nuclei were counted. In the absence of Rb, cells from the ganglionic eminence display more significant adherence to netrin-1 than cells from control littermates. (B) Fold increase represents the increase in adhesion from noncoated to netrin-1-coated wells. Error bars represent standard error of the mean ($n = 4$ for the controls and $n = 3$ for conditional Rb mutants). Significance was determined through a paired two-tailed t test for control and conditional Rb mutant cultures. *, $P < 0.05$.

that Rb-deficient neural precursor cells have increased adhesive properties, consistent with previous findings revealing a role for netrin-neogenin in mediating cellular adhesion (50, 51). Given the elevated levels of *neogenin* expression detected, increased adhesion in response to netrin-1 may be a key contributing factor to the migration defect present in Rb-deficient brains.

Increased neogenin impedes neuronal migration. While we have demonstrated that Rb is capable of regulating neogenin transcription through E2F in the developing nervous system, the consequence of increased neogenin expression remains unknown. We therefore asked if upregulation of neogenin as found in the Rb-deficient forebrain was sufficient to disrupt the migration of MGE-derived neurons. To determine whether increased neogenin expression could perturb neuronal migration, we performed *ex vivo* cortical electroporation of the full-length neogenin or a control internal ribosome entry site (IRES)-GFP vector into the ventral telencephalon of wild-type E15.5 embryonic brains (22). Following electroporation, brains were cultured as slices for 72 h to observe migration. Expression of the plasmid carrying GFP-positive cells was first observed at 24 h postelectroporation and subsequently at 72 h. At 24 h, brains electroporated with either control or neogenin-containing plasmids displayed GFP-positive cells lining the ventricular zone of the ventral forebrain, with no difference observed between controls and neogenin-electroporated cells (Fig. 6A, red). At 72 h, numerous GFP-positive cells from control slices were observed to have migrated considerably from their initial position within the ventricular zone. GFP-positive cells from neogenin slices, however, remained clustered within a similar band along the ventricular zone (Fig. 6A, green). Migration was quantified by measuring the total area occupied by GFP-positive cells at the endpoint, subtracting the initial area, and then dividing this value by the initial area to obtain the percent increase in migration [(total migration − initial migration)/total migration], and values were normalized to the percentage of the control. Upregulation of neogenin resulted in a 77% decrease in migration ($P < 0.05$) compared to that of control-electroporated embryos (Fig. 6B). We conclude that increased expression of neogenin by cells in the ganglionic eminence results in reduced migration of precursors away from the ventricular zone, paralleling the migration defect observed in the Rb-deficient forebrain.

Taken together, our results demonstrate a function for the

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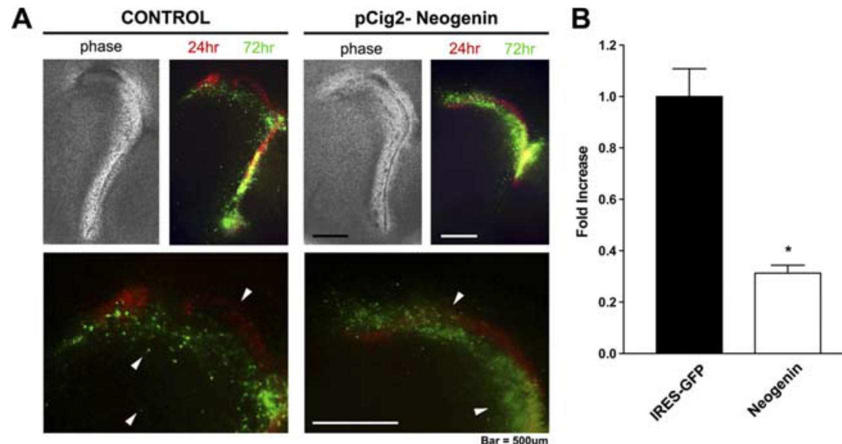

FIG. 6. Increased neogenin expression impairs migration of neuroblasts from the subventricular zone. (A) *Ex vivo* overexpression of control IRES-GFP- or neogenin-IRES-GFP-expressing plasmids in E15 embryos. Embryos were sectioned at 250 μm and plated on poly-L-lysine–laminin-coated inserts. Cells were visualized at 24 h postelectroporation (red) to determine their baseline vector expression and migration. Cells were imaged again at 72 h postelectroporation to assess the degree to which they migrated (green). Under both control and neogenin-overexpressing conditions cells expressing the plasmids initially line the ventricle (red, arrows). After migration (lower panels) the cells move away from the ventricular zone into the striatum (green, arrows) in the control; however, in the mutant they fail to shift position. (B) Quantification of the capacity of cells to migrate depicted in panel A. In order to quantify migration, fold increase was obtained by calculating (total migration − initial migration)/total migration and then normalizing values to wild-type migration levels. Error bars represent standard error of the mean ($n = 5$ for the control and $n = 6$ for the mutant, with two sections per embryo). Significance was determined through a paired two-tailed t test of control versus neogenin-overexpressing slices. *, $P < 0.05$.

Rb pathway in regulating expression of a nontraditional E2F target gene, neogenin, during neuronal migration. Furthermore, we demonstrate that aberrant neogenin expression, similar to that found in conditional Rb mutants, leads to impaired migration. Overall, these finding support the conclusion that Rb/E2F regulation of *neogenin* expression, an atypical target, influences appropriate neural development in a manner beyond traditional regulation of the cell cycle.

DISCUSSION

Here, we demonstrate the existence of an Rb/E2F-mediated molecular mechanism regulating expression of an atypical E2F target gene, *neogenin*. First, we have shown that *neogenin* expression is deregulated in the absence of Rb at the mRNA and protein levels in neural precursor cells. While neogenin has previously been shown to be an E2F-regulated target gene *in vitro*, here we complement previous findings by demonstrating that E2F3 is capable of activating neogenin expression, and we extend these findings by demonstrating the binding of E2F3 to the 5′ regulatory region of the neogenin promoter in neural precursor cells. It is possible that other E2Fs are contributing to regulation of neogenin expression in different biological contexts; however, given that E2F3 has previously been implicated in multiple aspects of nervous system development *in vivo* (9, 34, 36), these observations lend further support to the idea that E2F3 regulation is significant in this context. Finally, we demonstrate that E2F transcriptional regulation of neogenin is, in turn, strongly repressed by Rb activity. These results, along with our data regarding increased neogenin expression among migrating neurons in the absence of Rb, suggest a direct role for the regulation of neogenin by the Rb/E2F pathway in the developing forebrain.

Previously, we have shown that the Rb/E2F pathway mediates migration of a population of precursors from the ventral telencephalon during nervous system development (16, 34). Here, we provide mechanistic insight into this process, showing that in the absence of Rb, migrating ventral precursors exhibit a decreased response to the neogenin ligand, netrin-1. Consistent with decreased migration, we observe increased adhesion of ventrally derived Rb-deficient precursors to substrate-bound netrin-1. This suggests a mechanism in which increased neogenin expression results in augmented neuronal adhesion leading to the decreased migration. Our *ex vivo* manipulation of neogenin expression resulted in a defect in neuronal migration similar to that seen in the conditional Rb mutant (16). While we favor our model as a hypothesis to explain how Rb/E2F regulates migration, we note that neogenin is likely only one of many factors contributing to Rb-mediated neuronal migration. Indeed, neuronal migration is a complex phenomenon involving multiple genes and genetic pathways (24). Through our previous microarray analysis we identified several known genes that regulate migration in the central nervous system, and therefore dysfunction in their expression could also be contributing to several facets of the observed migration defect in the conditional Rb mutants (34). While the extent to which deregulated neogenin contributes to the migration defect in Rb mutants is unknown, our studies reveal that overexpression of

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neogenin perturbs neuroblast migration in wild-type tissue (Fig. 6). Indeed, a rescue experiment in our conditional Rb mutants would be challenging as reducing neogenin expression to physiological levels without causing a complete knockdown would likely lead to variable results. As presented, our results provide strong evidence that, by regulating neogenin expression, Rb/E2F has an important physiological role beyond regulation of the cell cycle machinery, a phenomenon that has not yet been reported. It is probable that Rb is involved in the regulation of multiple genes, which through distinct mechanisms contribute to the regulation of neuronal migration.

The idea that the Rb/E2F pathway can regulate genes outside the prototypical cell cycle machinery in the context of nervous system development may also broaden its role in tumorigenesis. As Rb is the first identified tumor suppressor, intense interest has been focused on defining the molecular mechanisms through which it mediates tumor suppression. While early studies established the model that Rb-mediated tumor suppression is the result of its restraint of E2F transcription factors at the G_1/S transition, (reviewed in reference 55), more recent studies suggest that the role of Rb as a tumor suppressor is more complex than originally hypothesized. Indeed, roles for Rb in maintaining genome stability and promoting senescence have broadened the scope and complexity of Rb-mediated tumor suppression (reviewed in references 21 and 30). Further deregulation of the Rb pathway in cancer has been traditionally associated with sustained proliferation; however, Rb mutations are frequently found in metastatic cancers, including small-cell lung carcinoma and osteosarcoma, as well as invasive poor-prognosis glioblastomas (reviewed in reference 12).

Having demonstrated a novel role for the Rb/E2F pathway in mediating expression of a specific gene involved in neuronal migration, the data presented here raise the possibility that Rb activity could contribute to the regulation of other cellular processes involved in cancer beyond regulation of cell division. Recent studies employing conditional transgenic alleles to remove tumor suppressor genes specifically in adult neural precursor cells have shown the important nonoverlapping roles for Rb, PTEN, Nf1, and p53 (1, 27). The study of Jacques et al. correlated the ablation of Rb gene expression in the adult subventricular zone with the appearance of primitive neuroectodermal tumors (pNETS). These tumors display significant differentiation across all three neural lineages and ectopic infiltration of surrounding brain tissue. This lends itself to the hypothesis that Rb may also regulate the differentiation and localization of these tumor cells. Many families of genes which mediate neuronal migration, such as the netrin signaling axis (18, 48), have been implicated in multiple aspects of cancer and tumorigenesis. Ligands and receptors from these migration pathways are frequently found deregulated or are lost altogether in numerous cancers (reviewed in references 2 and 8). Our findings demonstrate a key role for Rb/E2F-regulated expression of neogenin. Contributing to neuronal migration gives rise to the possibility that Rb-mediated mechanisms may regulate expression of migration-related genes during steady-state events such as neurogenesis. The deregulation of these processes may contribute to facets of tumor progression that expand from the typical aberrant S-phase entry associated with Rb loss of function. Further exploration of this hypothesis in the context of tumorigenesis could lend new insight into our understanding of the mechanisms of Rb-mediated tumor suppression.

In conclusion, our results suggest that Rb/E2F is required for the regulation of neogenin during neuronal migration. Further, these results provide strong support to our overall hypothesis that Rb acts through E2F to mediate events distinct from cell cycle progression by regulating transcription of genes that are not classical E2F targets.

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APPENDIX D- CO-AUTHOR PUBLICATIONS – FIRST PAGE

Cell Stem Cell
Article

Opposing Regulation of *Sox2* by Cell-Cycle Effectors E2f3a and E2f3b in Neural Stem Cells

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SUMMARY

The mechanisms through which cell-cycle control and cell-fate decisions are coordinated in proliferating stem cell populations are largely unknown. Here, we show that E2f3 isoforms, which control cell-cycle progression in cooperation with the retinoblastoma protein (pRb), have critical effects during developmental and adult neurogenesis. Loss of either E2f3 isoform disrupts *Sox2* gene regulation and the balance between precursor maintenance and differentiation in the developing cortex. Both isoforms target the *Sox2* locus to maintain baseline levels of *Sox2* expression but antagonistically regulate Sox2 levels to instruct fate choices. E2f3-mediated regulation of *Sox2* and precursor cell fate extends to the adult brain, where E2f3a loss results in defects in hippocampal neurogenesis and memory formation. Our results demonstrate a mechanism by which E2f3a and E2f3b differentially regulate *Sox2* dosage in neural precursors, a finding that may have broad implications for the regulation of diverse stem cell populations.

INTRODUCTION

Stem cell-fate decisions, such as self-renewal, precursor cell maintenance, and commitment to differentiation have critical outcomes for embryonic development, tissue maintenance, tumor suppression, and regeneration. Cortical development depends on a precisely regulated balance of self-renewal within stem cell-like apical precursors (APs), production of rapidly proliferating basal progenitors (BPs), and differentiation of postmitotic neurons (Englund et al., 2005; Farkas and Huttner, 2008; Hutton and Pevny, 2011) (Figure 1A). Identifying mechanisms that control this balance can inform our understanding of developmental neurogenesis and, more broadly, reveal stem cell biological principles extending to embryonic stem cell differentiation, tumor formation, and tissue regeneration.

The pluripotency factor Sox2 is an established regulator of neural precursor proliferation, self-renewal, and differentiation during development and is also required for maintenance of adult stem cell populations in many different tissues (reviewed in Sarkar and Hochedlinger, 2013). Overexpression of Sox2 in both mouse and chick embryonic neural precursor cells (NPCs) results in maintenance of the Sox2^{+} population and defective neurogenesis (Bani-Yaghoub et al., 2006; Graham et al., 2003). Conversely, loss of function of Sox2 in neural precursors leads to precursor loss and reduced or aberrant differentiation, depending on the tissue type and degree of Sox2 loss (Cavallaro et al., 2008; Favaro et al., 2009; Ferri et al., 2004; Graham et al., 2003; Miyagi et al., 2008; Taranova et al., 2006). Taken together, these studies reveal that the function of Sox2 is strongly influenced by dosage; thus, fine-tuning of transcription from the *Sox2* locus is crucial for the generation of the correct proportion of precursors versus differentiated cell types. Interestingly, a recent study finds that the Cyclin-dependent kinase inhibitor 1A (p21) binds a *Sox2* enhancer region to regulate Sox2 expression and adult neurogenesis, linking cell-cycle regulation with Sox2-mediated control of neural stem cell (NSC) expansion (Marqués-Torrejón et al., 2013).

Previous evidence suggests that the cell cycle machinery plays a key role in regulating the proliferative expansion and self-renewal capacity of NPCs (Nishino et al., 2008; Ruzhynsky et al., 2007; Vanderluit et al., 2004). However, how specific cell-cycle regulatory proteins function in this context remains poorly defined. The retinoblastoma pocket protein (pRb) family controls cell-cycle progression by binding and inhibiting the E2f family of transcription factors. E2fs are classified into the "activator" subclass, which drives proliferation and transcription, and the "repressor" subclass, the members of which are thought to repress gene transcription by modifying chromatin structure through association with pocket proteins (Asp et al., 2009). Earlier work has reported that E2f3 is the most highly expressed E2f family member in wild-type and pRb-deficient neural precursors (Callaghan et al., 1999), suggesting that it may be an important regulator of NPC functions. Understanding how the *E2f3* gene functions to regulate the cell cycle is not entirely straightforward, because the two isoforms (*E2f3a* and *E2f3b*) expressed from its locus have identical domains important for DNA binding, transactivation, and pocket-protein binding, and only their N termini are unique. Mice lacking both

Development/Plasticity/Repair

The Rb/E2F Pathway Modulates Neurogenesis through Direct Regulation of the *Dlx1/Dlx2* Bigene Cluster

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During brain morphogenesis, the mechanisms through which the cell cycle machinery integrates with differentiation signals remain elusive. Here we show that the Rb/E2F pathway regulates key aspects of differentiation and migration through direct control of the Dlx1 and Dlx2 homeodomain proteins, required for interneuron specification. Rb deficiency results in a dramatic reduction of *Dlx1* and *Dlx2* gene expression manifested by loss of interneuron subtypes and severe migration defects in the mouse brain. The Rb/E2F pathway modulates *Dlx1/Dlx2* regulation through direct interaction with a *Dlx* forebrain-specific enhancer, I12b, and the *Dlx1/Dlx2* proximal promoter regions, through repressor E2F sites both *in vitro* and *in vivo*. In the absence of Rb, we demonstrate that repressor E2Fs inhibit *Dlx* transcription at the *Dlx1/Dlx2* promoters and *Dlx1/2*-I12b enhancer to suppress differentiation. Our findings support a model whereby the cell cycle machinery not only controls cell division but also modulates neuronal differentiation and migration through direct regulation of the *Dlx1/Dlx2* bigene cluster during embryonic development.

Introduction

During brain development, cell cycle regulation and differentiation are tightly coordinated developmental programs. Cross talk exists between the cell cycle machinery and differentiation pathways to ensure that progenitor populations are maintained and that differentiation is induced at the time of terminal mitosis (McConnell and Kaznowski, 1991; Nguyen et al., 2006; Farkas and Huttner, 2008; Frank and Tsai, 2009). Despite the importance of the precise coordination of these events, the mechanisms by which the cell cycle machinery integrates with differentiation signals remain poorly understood.

The retinoblastoma protein, pRb, is a tumor suppressor gene that controls the G_1-S phase checkpoint during cell cycle regulation (McClellan and Slack, 2006; Chen et al., 2009; Freedman et al., 2009). Rb regulates the transcription of genes that are required for DNA replication and cell cycle progression by binding and inhibiting E2F transcription factors (Burkhart and Sage, 2008). There are eight E2Fs, five of which can bind Rb (E2F1–5) and are considered among the classical Rb partners while E2F6 – 8 are Rb-independent repressors (Dick and Dyson, 2006; Chen et al., 2009; Lammens et al., 2009). E2F1, 2, and 3 are primarily transcriptional activators while E2F4 and 5 repress transcription and induce gene silencing through pocket protein binding (Dick and Dyson, 2006). E2F7 and E2F8, two atypical E2Fs (Lammens et al., 2009), can form homo and heterodimers which, in the absence of pocket proteins, bind and repress E2F target genes. The expression of E2F7 and 8 is induced by activating E2Fs and are believed to serve as a fine tuning mechanism to modulate E2F target gene regulation (Di Stefano et al., 2003; Christensen et al., 2005; Lammens et al., 2009).

It has been proposed that the Rb pathway may have novel function(s) that extend beyond cell cycle control (McClellan and Slack, 2006, 2007). Conditional knock-out studies have suggested that Rb may have a role in regulating differentiation and migration (Takahashi et al., 2003; Ferguson et al., 2005; Chen et al., 2007; McClellan et al., 2007); however, the underlying mechanisms remain unknown. Clearly, indirect cross talk between the cell cycle machinery and differentiation pathways is essential to prevent premature differentiation of proliferating progenitors while promoting differentiation as cell division ceases. If the cell cycle proteins themselves could directly regulate genes required for differentiation, these two processes could become intimately coordinated.

Here we have uncovered a more direct role for cell cycle proteins in neuronal differentiation through the control of Dlx1 and Dlx2 homeodomain protein regulation, two key proteins that specify GABAergic neurons in the brain. Consistent with a deficit in *Dlx1/Dlx2* gene expression, mice lacking Rb exhibited a pro-

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The p107/E2F Pathway Regulates Fibroblast Growth Factor 2 Responsiveness in Neural Precursor Cells[▿]

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We have previously shown that p107, a member of the retinoblastoma (Rb) cell cycle regulatory family, has a unique function in regulating the pool of neural precursor cells. As the pool of progenitors is regulated by a limiting supply of trophic factors, we asked if the Rb/E2F pathway may control the size of the progenitor population by regulating the levels of growth factors or their receptors. Here, we demonstrate that fibroblast growth factor 2 (FGF2) is aberrantly upregulated in the brains of animals lacking Rb family proteins and that the gene encoding the FGF2 ligand is directly regulated by p107 and E2F3. Chromatin immunoprecipitation assays demonstrated that E2F3 and p107 occupy E2F consensus sites on the FGF2 promoter in the context of native chromatin. To evaluate the physiological consequence of FGF2 deregulation in both p107 and E2F3 mutants, we measured neural progenitor responsiveness to growth factors. Our results demonstrate that E2F3 and p107 are each mediators of FGF2 growth factor responsiveness in neural progenitor cells. These results support a model whereby p107 regulates the pool of FGF-responsive progenitors by directly regulating FGF2 gene expression in vivo. By identifying novel roles for p107/E2F in regulating genes outside of the classical cell cycle machinery targets, we uncover a new mechanism whereby Rb/E2F mediates proliferation through regulating growth factor responsiveness.

Cell cycle genes have been found to play an important role in brain development, with numerous molecules regulating the G_1/S transition having been shown to regulate neural precursor proliferation (reviewed in reference 38). Perhaps the most important regulators of the G_1/S transition are the retinoblastoma protein (Rb) and its closely related family member p107. Rb is a pivotal regulator of neural precursor proliferation and the timing of cell cycle withdrawal. For example, Rb has been shown to regulate terminal mitosis of neuroblasts in the central and peripheral nervous systems and retina (7, 18, 34, 35). Furthermore, recent evidence has emerged indicating that Rb itself is capable of regulating diverse cellular processes in the nervous system beyond proliferation. Roles for Rb have been indicated in laminar patterning of the cortex and neuronal migration (17; reviewed in reference 38). These studies highlight the importance of Rb in regulating neural cell populations. In contrast to Rb, little is known about the role of p107. While its role was originally thought to overlap with and compensate for that of Rb (29), distinct functional differences in tissues such as muscle, chondrocytes, and adipocytes, have emerged, suggesting otherwise (10, 28, 51). We have recently shown that p107 plays a unique role, one distinct from Rb, in regulating neural precursor cell numbers in the developing and adult brain (60). p107 null neural precursor cells have an enhanced capacity for self-renewal and, consistent with this, exhibit expanded populations of both precursors and progenitors. While we have previously demonstrated that the increased self-renewal capacity and neural precursor numbers are due, in part, to an upregulation of the Notch-Hes signaling pathway (61), the mechanisms that sustain the increased population are still unknown.

The E2F family of transcription factors, comprised of E2F1 to E2F8, are key Rb/p107-interacting targets best known for their role in promoting cell cycle progression (reviewed in reference 59). Accumulating in vitro and in vivo evidence, however, suggests that E2Fs are capable of regulating expression of a broad spectrum of genes and diverse physiological processes (reviewed in reference 39). In vitro, microarray studies examining changes in gene expression in response to various models of deregulated E2F expression have each identified groups of overlapping novel target genes with well-characterized roles in differentiation, development, and migration (3, 12, 25, 33, 41, 43, 68). Chromatin immunoprecipitation (ChIP)-on-chip studies have localized E2Fs to a number of gene promoters unrelated to cell cycle (1, 2, 6, 26, 47, 64, 65). In vivo, E2Fs have been implicated in a number of distinct aspects of nervous system development. E2F4 has been shown to regulate development of the ventral telencephalon through a genetic interaction with the Sonic hedgehog pathway (50), while E2F1 and E2F3 have been implicated in mediating neural precursor proliferation (11, 37). Intriguingly, in vivo models are emerging to suggest that Rb family members interact with E2Fs to mediate novel functions in nervous system development. For example, Rb has been shown to interact with both E2F3 and E2F1 to mediate neural precursor proliferation and cell cycle exit (8, 37). Additionally, Rb has been shown to mediate neural migration and differentiation, in a manner beyond cell cycle regulation, uniquely through E2F3 (8, 37). Given the emerging

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